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ACTION OF PYRIDINE AND SOME DERIVATIVES OF PYRIDINE-3-CARBOXYLIC ACID ON NEUROMUSCULAR TRANSMISSION

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(1) In an earlier publication from this department (Huidobro and Jordan), it was shown that the diethylamine of pyridine-3-carboxylic acid (nikethamide) reinforces the action of curare as well as the action of prostigmine upon the neuromuscular preparation. In view of the antagonism between these two drugs, these findings suggested an investigation of the pharmacologic action of other pyridine structures on neuromuscular transmission.

The present study was undertaken to determine whether pyridine or some of its derivatives enhance the action of curare and prostigmine as well as to study further the pharmacological action of the diethylamine of pyridine-3-carboxylic acid (DA). Therefore, a comparative study has been made of the action of pyridine (P), pyridine-3-carboxylic acid (niacin, N), the amide of pyridine-3-carboxylic acid, (niacnamide, NA), the ethyl ester of pyridine-3-carboxylic acid (EE), the monoethylamine (MA) and the diethylamine (DA) of the same acid

METHODS The animals used were cats anesthetized with sodium pentobarbital (nembutal, Abbott, 0.033 Gm dissolved in 1 cc of 25% urethane, per kilogram body weight, intraperitoneally). The principal muscle studied was the quadriceps femoris, a few of the observations were made on the soleus. Both muscles were stimulated indirectly through the nerves by condensers, which in turn were controlled by vacuum tubes. Maximal stimuli were applied. The electrodes were made of silver, insulated with rubber. A record of muscular response was obtained by holding the femur in fixed clamps, with the corresponding tendon attached to the short end of a recording lever, pulling against elastic bands.

In some experiments the homologous contralateral muscle was stimulated directly, being used 5 to 8 days after its denervation. In these cases steel needle electrodes were inserted into the tendon and into the muscle belly. The stimulus was obtained from a Harvard inductorium, the primary circuit of which received a 3 volt current of 50 cycles per minute, though at times a 10 volt current was used. The distance between primary and secondary circuits was that necessary for the production of maximal stimuli.

In other experiments the muscle was stimulated directly with acetylcholine. In these the soleus was used, having been denervated aseptically 5 to 12 days previously. The stimulating dose of acetylcholine varied between 20 and 200 micrograms, usually between 20 and 30 micrograms were given. This drug was administered in an aqueous solution not exceeding a volume of 0.20 cc, by injection into the abdominal aorta under the conditions described later in this paper.

The drugs employed were pyridine (Baker's), pyridine-3-carboxylic acid (Sanitas), the amide of pyridine 3 carboxylic acid (Laboratorio Chile), the ethyl ester of pyridine 3-carboxylic acid, the monoethylamide and diethylamide of pyridine-3 carboxylic acid (coramine, Ciba). All of these drugs were administered as a 1 M solution, except for DA

which was occasionally given in a 25% solution. The following substances were also used: prostigmine (Roche) in 0.05% solution, curare (crude product from Brazil), potassium chloride (Merek) in 20% solution, atropine sulfate (Merek) in 1% solution, acetylcholine hydrochloride (Roche) in the quantities stated under "Results." All of these drugs were dissolved in distilled water. The niacin was neutralized with sodium hydroxide.

With the exception of atropine, which was given intravenously, the drugs were administered by injection into the terminal end of the abdominal aorta after a preliminary ligation of the inferior mesenteric artery and the middle sacral artery and, in those cases in which the muscle of only one extremity was being observed, the contralateral iliac artery.

In studies upon the action of the pyridine derivatives on the activity of choline esterase, the determinations were made on the blood serum according to the technique of Pighini (2), modified as follows: to 0.25 cc. of human serum, free of hemolysis, was added 25 cc. of distilled water, 20 milligrams of acetylcholine, 3 drops of phenolphthalein, and 0.10 cc. of a molar solution of the drug under investigation. Titration was carried out at room temperature (18°C) with N/200 sodium hydroxide, which was added every 3 minutes for 15 minutes. The results were expressed in cc. of N/200 sodium hydroxide used during 15 minutes.

RESULTS. I. *Action of P, N, NA, EE, MA, and DA on indirect muscular stimulation.* A uniform volume (0.25 cc.) of equimolecular solutions of these drugs, was injected into the abdominal aorta while the muscle was stimulated indirectly.

When frequencies fluctuating between 100 and 140 stimuli per minute were used, the drugs P, N, NA, and MA did not produce any variation in the tension of muscular contraction (figures 1, 2, 3, and 6). EE produced a definite depression of short duration. Repeated injections of this drug did not have a cumulative effect, (lower section of figure 4.) This contrasts with the previous results obtained with DA, since the latter substance produced an increase in the tension developed (Huidobro and Jordan, 1).

When frequencies varying between 30 and 75 stimuli per second were used, all of the drugs except NA had a significant effect. With P, an increase in tension resulted in 8 out of 12 experiments, as shown in figure 1A. In the other four experiments there was a depression. The drug N consistently produced (in 10 cases) an increase in tension, which differed from that observed with P in that it was less and of shorter duration. This increase was followed by a phase of depression, from which there was recovery after one minute (figure 2A). NA, in the 8 experiments in which it was used, did not produce any significant change (figure 3A). EE consistently produced an intense depression of the tension, which did not last longer than one minute. In 6 of the 14 experiments, the tension, subsequent to this depression, increased to a level greater than that prior to injection. This substance, at these frequencies of stimulation, resembles DA in its action. However, the depression produced by DA is more prolonged and is not followed by an increase of tension. MA produced a depression very similar to that of DA. However, in half of the experiments, the tracing resembled the curve of a hook which was of lesser amplitude and more open than the hook produced by EE at a higher frequency (figure 5A).

When the muscle was stimulated indirectly at a higher frequency, (250-500 per second) and the 3c phase appeared (Rosenblueth and Cannon, 3), P consistently produced an abrupt and brief fall in the tension, followed by an immediate recovery, which in all 22 experiments, reached a level higher than that observed

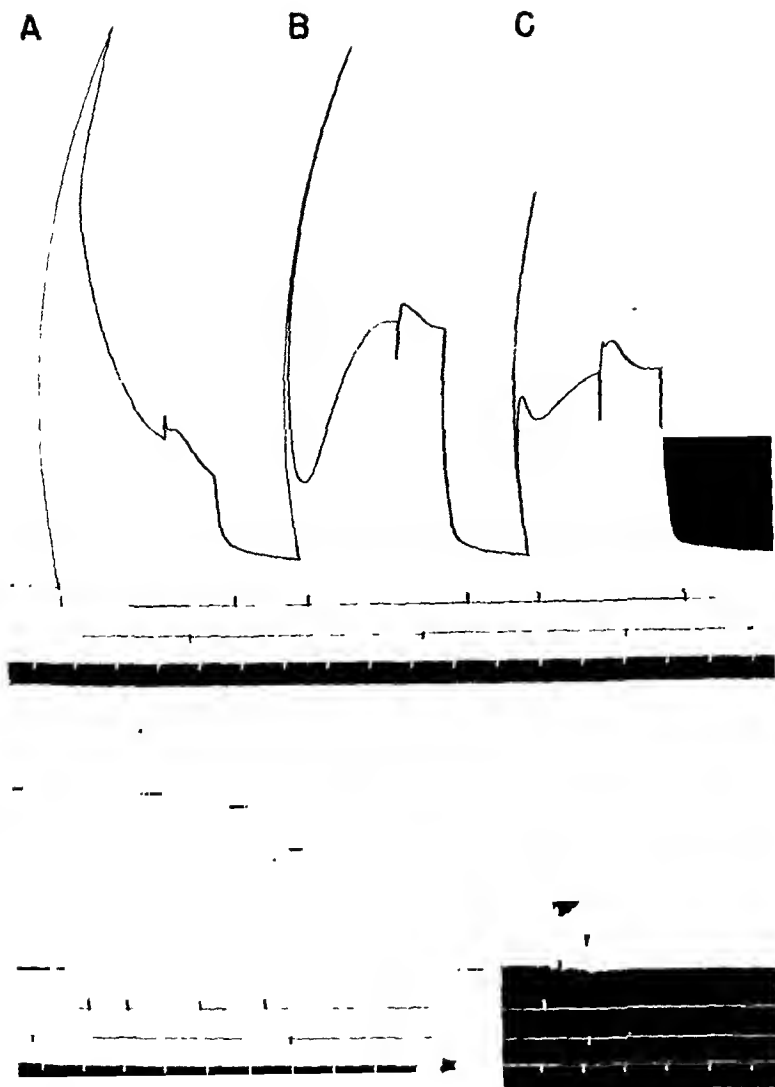


FIG. 1

Action of Pyridine (P) on the contraction of the quadriceps muscle produced by indirect stimulation, and on the effect of curare and prostigmine. Cat anesthetized with nembutal. Upper tracing: A. Quadriceps muscle stimulated indirectly at a frequency of 50 per second. Signals on upper line: beginning and end of stimulation. Signals on middle line: 0.25 c.c. of P. Lower line, time in minutes (same in all figures).

B. Same as A, with muscle stimulated at 250 per second.

C. Same as A, with muscle stimulated at 500 per second.

Lower left tracing: Stimulation of the muscle at a frequency of 120 per minute. Signals on upper line: curare. Signal on middle line: P.

Lower right tracing: Stimulation of the muscle at a frequency of 100 per minute. Signals on upper line: 360 micrograms of prostigmine. Signals on middle line: P.

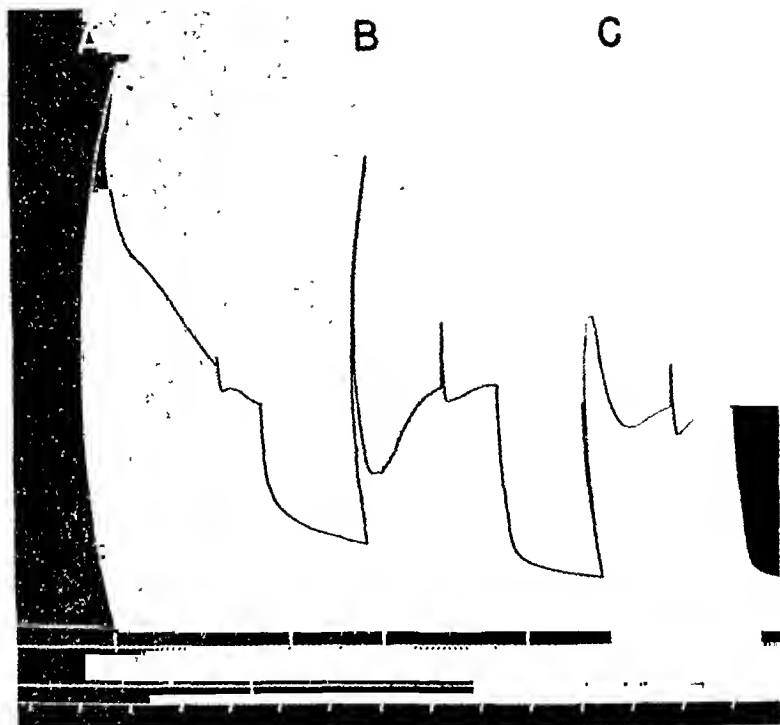


FIG. 2

Action of Pyridine-3-carboxylic acid (N) on the contraction of the quadriceps stimulated indirectly, and on the effect of curare and of prostigmine. Cat anesthetized with nembutal.

Upper tracing: A. Stimulation of the muscle at a frequency of 50 per second. Signals on upper line; beginning and end of stimulation. Signal on middle line: N.

B. Same as A, with muscle stimulated at 250 per second.

C. Same as A, with muscle stimulated at 500 per second.

Lower left tracing: Muscle stimulated at a frequency of 100 per minute. Signals on upper line; curare. Signal on middle line: N.

Lower right tracing: Same animal as left tracing and same frequency of stimulation. Signal on upper line: 375 micrograms of prostigmine. Signal on middle line: N.

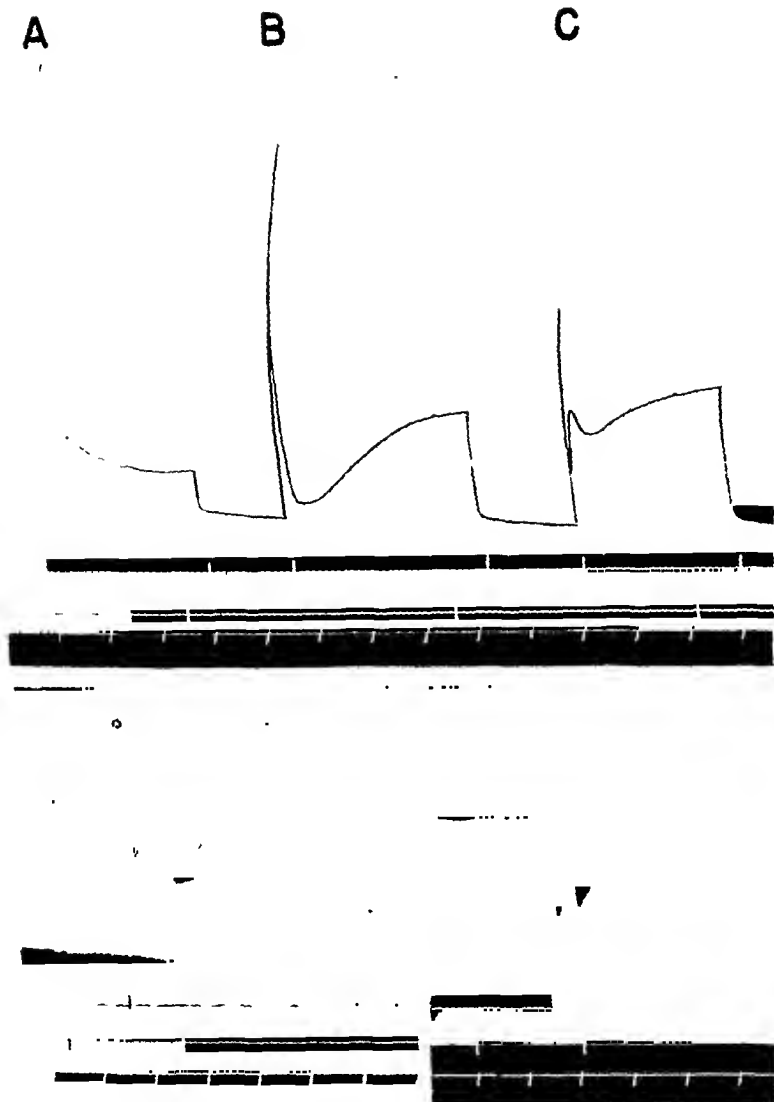


FIG. 3

Action of the amide of pyridine-3-carboxylic acid (NA) on the contraction of the quadriceps muscle produced by indirect stimulation, and on the effect of curare and of prostigmine. Cat anesthetized with nembutal.

Upper tracing: A. Stimulation of muscle at a frequency of 50 per second. Signals on upper line: beginning and end of stimulation. Signals on middle line: NA.

B. Same as A, with muscle stimulated at 250 per second.

C. Same as A, with muscle stimulated at 500 per second.

Lower left tracing: Muscle stimulated at a frequency of 120 per minute. Signals on upper line: curare. Signals on middle line: NA.

Lower right tracing: Same animal and same frequency of stimulation as left tracing, 31 minutes later. Signal on upper line: 350 micrograms of prostigmine. Signals on middle line: NA.

prior to injection. As shown in fig. 1, the effects of the drug P vary according to the frequency of stimulation. In the case of N, there occurred in all 24 experiments, an increase in tension, followed by a phase of depression (figures 2B and C). NA did not produce any changes, in 14 experiments (figures 3B and C). The drug EE again produced a profound depression. However, in all 16 experiments there was a fluctuation of the tension, which gave to the tracing an appearance of a W, the first two arms of which are very close together (figure 4B and C). Since EE is not water soluble and forms an oily emulsion, control experiments were done with an emulsion of olive oil. This was found to have no effect upon the curve of muscular contraction. The drug MA consistently produced a decrease in the tension in which there again appeared the W configuration, (figure 5B and C).

II. *The Effects of P, N, NA, EE, and MA on the Action of Curare and of Prostigmine during Indirect Muscular Stimulation.* This study was carried out upon the quadriceps muscle, stimulated through its nerve at a frequency which varied between 100 and 140 per minute. The dose of curare was never large enough to necessitate artificial respiration. The prostigmine was injected in a dose which varied between 125 and 375 micrograms; however, at times it was necessary to repeat the dose in order to achieve a greater prostigminization of the animal. The drugs studied were injected in a volume of 0.25 cc. of a IM solution following either curare or prostigmine. The results fall into two groups, as follows:

A. P and NA. These substances have a decurarizing effect and appear to reinforce the action of prostigmine, but these effects are not intense, (figures 1 and 3, lower section).

B. N, EE, and MA. N has a slight decurarizing effect and is slightly antagonistic to prostigmine (figure 2, lower section). The former effect is consistently more in evidence than the latter. EE reinforces the action of curare, as well as that of prostigmine (figure 4, lower section). Huidobro and Jordan (1946) found a similar action for DA. The drug MA also appears to enhance the action of both curare and prostigmine, but to a small degree. In order to demonstrate this reinforcing action, it is necessary for the animal to have received a large dose of prostigmine (figure 6A) and also that the dose of curare be great enough to prevent muscular contraction by indirect stimulation (figure 6B).

III. *Action of P, N, and EE on Muscular Contraction by Direct Stimulation.* In this study, simultaneous recordings of the contractions of both solcus muscles or of both quadriceps muscles were made; one was stimulated indirectly, the other directly. The latter had been denervated 5 to 8 days previously. This was considered a sufficient interval for the degeneration of the nerve (Lisak, Dempsey, and Rosenbluth, 4). Stimulation, both direct and indirect, was applied with a frequency of 50 stimuli per second for 3 seconds; a rest period of 57 seconds was allowed between stimuli. When a series of contractions of equal amplitude was obtained, the drug was injected 10 to 15 seconds prior to a contraction. At the end of the experiment, a tetanic stimulus was applied to the muscle, and the drug injected during the tetanus. The doses employed were

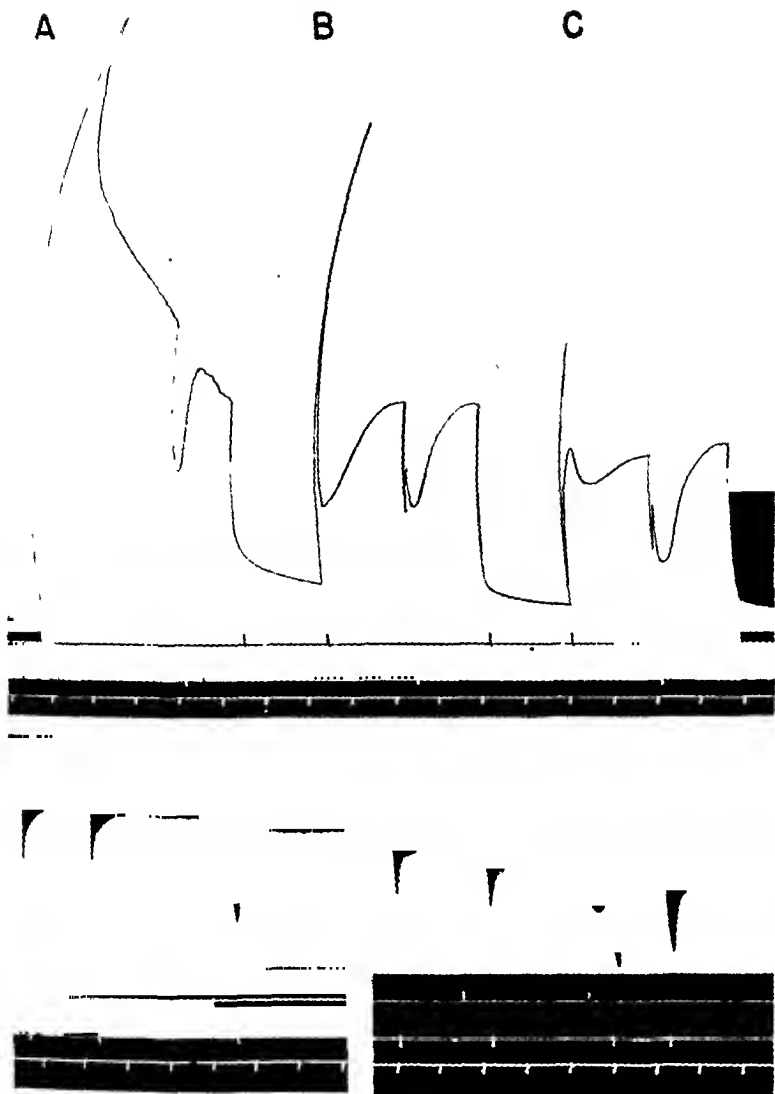


FIG. 4

Action of the ethyl-ester of pyridine-3-carboxylic acid (EE) on the contraction of the quadriceps muscle produced by indirect stimulation, and on the effect of curare and prostigmine. Cat anesthetized with nembutal.

Upper tracing: A. Stimulation of muscle at a frequency of 50 per second. Signals on upper line: beginning and end of stimulation. Signal on middle line: EE.

B. Same as A, with muscle stimulated at 250 per second.

C. Same as A, with muscle stimulated at 500 per second.

Lower left tracing: Muscle stimulated at a frequency of 120 per minute. Signal on upper line: curare. Signals on middle line: EE.

Lower right tracing: Same animal and same frequency of stimulation as left tracing, 39 minutes later. Signals on upper line: 325 and 375 micrograms of prostigmine respectively. Signals on middle line: EE.

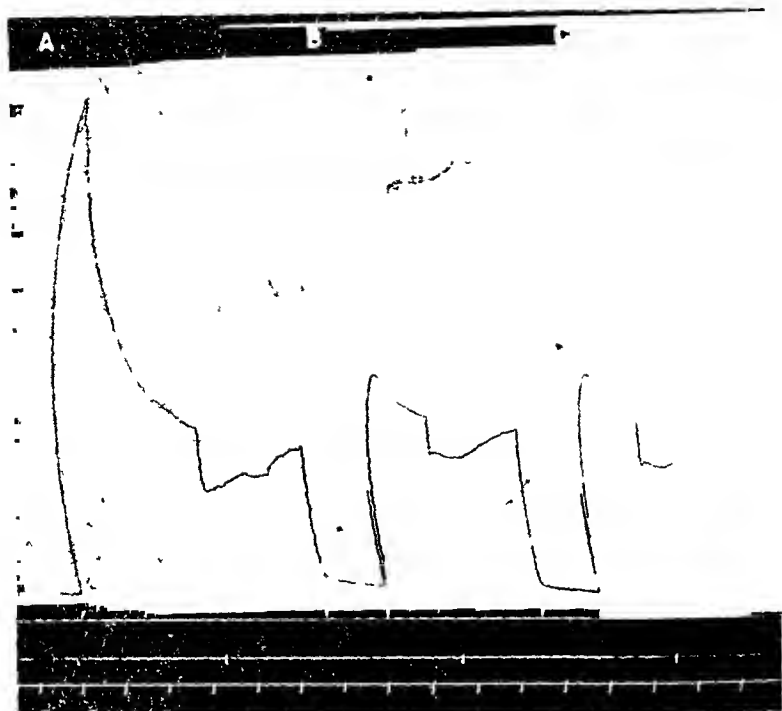


FIG 5

Action of the monoethyl amide of pyridine 3 carboxylic acid (MA) on the contraction of the quadriceps muscle produced by indirect stimulation. Cat anesthetized with nembutal.

A, B, and C Stimuli at a frequency of 50, 250, and 500 per second, respectively. Signals on upper line beginning and end of stimuli. Signals on middle line MA.

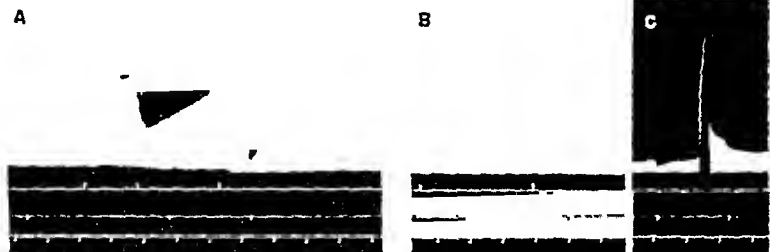


FIG 6

Action of the monoethyl amide of pyridine-3 carboxylic acid (MA) on the effect of curare and of prostigmine. Cat anesthetized with nembutal.

A Muscle stimulated at a frequency of 110 per minute. Signals on upper line 300, 250, and 350 micrograms respectively of prostigmine. Signals on middle line MA.

B Same animal and same stimuli as A, 30 minutes later. Signals on upper line curare. Signals on middle line MA.

C Six minutes later than B. Signals on middle line MA (the increase of tension produced by the muscle is due to a tetanic stimulation of 20 seconds at a frequency of 150 per second).

greater than those used in the other experiments, being 0.30 cc. of the molar solutions.

The injection of P was without effect when administered immediately before the short tetanus; when it was injected during the prolonged tetanic contraction, it produced a slight depression. The drug N was without effect. The effect of EE was similar to that of P, except that the depressant effect was greater when

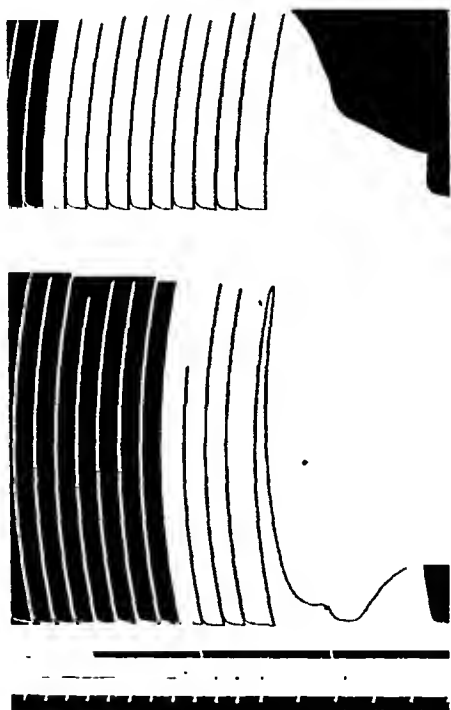


FIG. 7

Action of the ethyl ester of pyridine-3-carboxylic acid (EE) on the muscular contraction of the solus muscle stimulated directly (above), and indirectly (below). Cat anesthetized with nembutal.

The upper muscle was denervated aseptically 6 days previously. First signal on upper line: 0.25 c.c. of EE; second and third signals on same line: 0.30 c.c. of EE.

injected during prolonged tetanus (figure 7). Similar effects were obtained with EE and DA, (Huidobro and Jordan, 1).

IV. *Effect of P, N, NA, and EE on the Response of Muscles Stimulated by Acetylcholine.* This study was carried out on the solus muscle, which had been denervated 5 to 12 days previously. Before the injection of acetylcholine, each animal received one mg. per Kg. of atropine intravenously. The acetylcholine was injected into the abdominal aorta in doses varying from 20 to 200 micro-

grams, the amounts most frequently used being 20 or 30 micrograms, dissolved in a volume of distilled water never greater than 0.20 cc. Injections were made every three minutes. When responses of equal amplitude were obtained, the pyridine derivative was injected in a dosage of 0.25 cc. of a molar solution immediately prior to an acetylcholine injection. Under these circumstances, the four substances investigated produced an increase in the amplitude of contraction. In some cases this was greater than 100% (figure 8). This reinforcement occurred in the contraction immediately following the injection of the drugs but did not appear in subsequent contractions. In some experiments, the drugs N and E produced an increase of a more lasting degree (figure 8, upper left hand portion). It is noteworthy that Huidobro and Jordan (1) observed a similar effect with DA.

V. *Effect of P, N, EE, and DA on the Action of Acetylcholine and Potassium Chloride Injected during Indirect Muscular Stimulation.* To confirm the results described in the preceding section, acetylcholine was injected during indirect muscular stimulation. It was found that, with a stimulation rate of 75 per second, the injection of DA enhanced the effect of acetylcholine; that is, the phase of increased tension, which is thought to be produced by acetylcholine under experimental conditions, reached a greater height (Rosenbluth, Lissak and Lanari, 5). Similar results were obtained with EE; no effects were obtained with P and N.

In order to determine whether the K^+ ion might play a role in the effects observed in the preceding experiments, a series of experiments were performed using KCl in place of acetylcholine. Figure 9 indicates clearly that DA enhances the effect of potassium injected during indirect stimulation. The results obtained with EE were suggestive but not definite. P and N were without effect.

VI. *The Action of P, N, NA, EE, and DA on Choline Esterase Activity.* The fact that certain of these substances enhance the effect of acetylcholine on striated muscle, led to a study of their effects on choline esterase activity. The results are shown in table 1. The data indicates that neither pyridine nor any of its derivatives modifies significantly the choline esterase activity of blood serum.

DISCUSSION. The principal object of this study was to compare P, N, NA, EE, MA, and DA, with respect to their effect upon neuromuscular transmission, and to learn whether a relationship between their pharmacological activity and chemical structure existed. For this reason, the drugs were used in equimolecular solutions. Hence, the results obtained correspond to the effect of 0.25 cc. of the molar solutions of the various substances.

All of these drugs have one action in common, namely, to strengthen the response produced by acetylcholine. Since this enhancement is not produced by means of the inhibition of cholinesterase, and is not produced by a direct effect on the muscle, it can be assumed that this effect is due to the contraction of a larger number of muscle fibers. This is based on the observation of a decrease in the excitatory and paralytic thresholds of acetylcholine. The concept of the two thresholds is well accepted and has explained many phenomena relative to the chemical transmission of the nerve impulse, (Rosenbluth and Morison, 6).

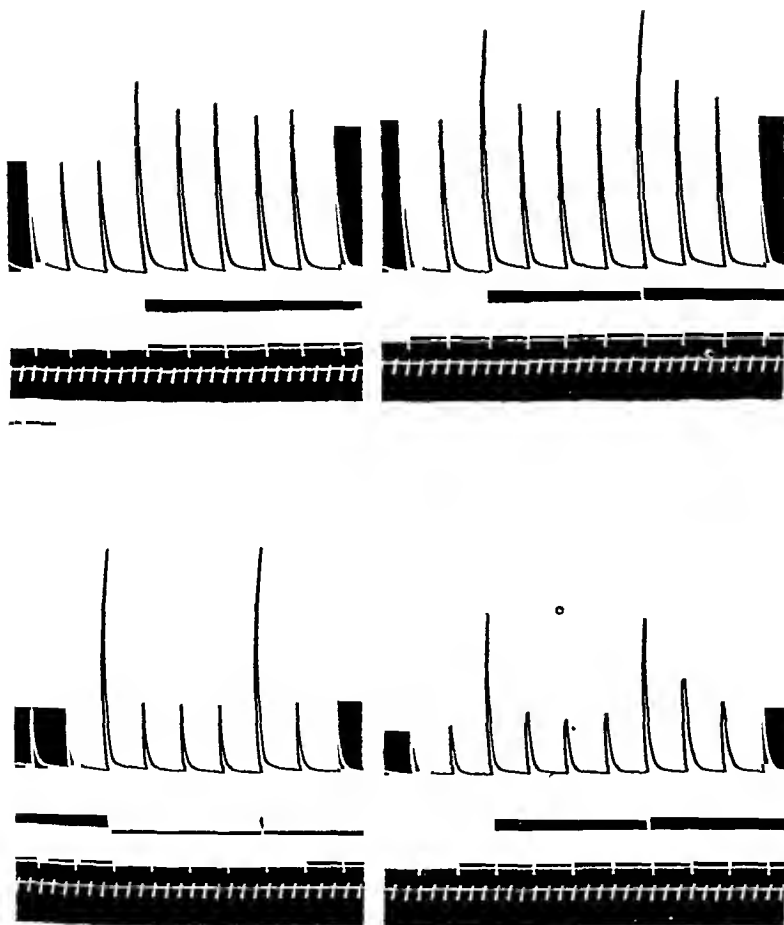


FIG 8

Action of N, EE, NA, and P on the responses of muscles stimulated by acetylcholine
Cat anesthetized with nembutal 1 mgm of atropine per kilogram, intravenously

Upper tracings Muscular contractions produced by 20 micrograms of acetylcholine
Soleus muscle denervated 8 days previously

Left Signals on upper line 0.25 c c of N Signals on middle line acetylcholine
Right Signals on upper line 0.25 c c of EE Signals on middle line acetylcholine
Lower tracings Muscular contractions produced by 80 micrograms of acetylcholine acting on the soleus muscle denervated aseptically 6 days previously

Left Signals on upper line 0.25 c c of NA Signals on middle line acetylcholine
Right Signals on upper line 0.25 c c of P Signals on middle line acetylcholine

All of the effects described for P and NA are readily understood if one accepts the concept that these two substances lower both the paralytic and the excitatory

thresholds of acetylcholine, and that P has in addition a slight depressant effect upon the muscle fiber itself. Actions of the drug N cannot be as readily explained. It would be logical to assume that it lowers the excitatory threshold but on this basis, its antagonism to prostigmine remains unexplained.

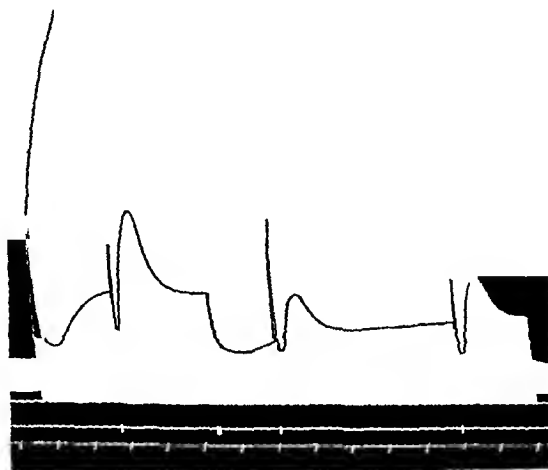


FIG. 9

Strengthening action of the diethyl amide of pyridine-3-carboxylic acid (DA) on the effect of potassium injected during indirect muscular stimulation. Cat anesthetized with nembutal. Quadriceps muscle stimulated indirectly at a frequency of 300 per second. The signals on the upper line indicate the beginning and end of stimulation. The thin signals on the middle line indicate the moment of the injection of 30 mgm. of potassium chloride. The thick signal indicates the moment of the injection of DA.

TABLE 1

The effect of pyridine derivatives on human serum esterase

	cc. N/200 NaOH					Mean
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	
Normal serum	2.27	1.14	2.04	1.99	1.75	1.84 ± 0.22
Normal serum with P	2.22	1.05	2.09	2.16	1.83	1.87 ± 0.21
Normal serum with N	2.33	1.17	2.02	2.18	1.90	1.92 ± 0.20
Normal serum with NA	2.27	1.02	1.98	2.22	1.90	1.89 ± 0.23
Normal serum with EE	2.02	1.02	2.10	1.80	1.62	1.71 ± 0.19
Normal serum with DA	2.23	0.95	2.06	2.00	1.71	1.79 ± 0.22

Results represent the average of duplicate titrations.

Huidobro and Jordan (1) came to the conclusion that DA decreased the excitatory threshold of acetylcholine, a concept which we have been able to corroborate. However, it does not have a decurarizing effect as might be expected from this theory; in fact, it reinforces the action of curare. In view of this,

one would expect DA to exert an antagonism toward prostigmine since curare and prostigmine manifest this. However, DA enhances the action of prostigmine. Furthermore, one cannot accept the idea that this drug lowers the paralytic threshold through facilitation of the action of acetylcholine, nor can its effects be explained in relation to potassium, since it similarly facilitates the action of this electrolyte.

For the same reasons, it is impossible to explain all of the actions of EE and MA as being effects upon the excitatory and paralytic thresholds of acetylcholine.

In summary, it is logical to suppose that pyridine and some of its derivatives disturb the chemical transmission of the nerve impulse, modifying in part the sensitivity of the effector to acetylcholine and in part acting through other mechanisms that are as yet not explained.

In reference to the relationships between the effects of the pyridine derivatives and their chemical structure, they have but one feature in common, namely, to lower the excitatory threshold of acetylcholine. Hence, it is concluded that the pyridine ring has the function of lowering the excitatory threshold of acetylcholine. The addition of a short lateral chain in position 3 and small modifications of this lateral chain alter the pharmacological action of pyridine. There are two groups of such modifications. The first involves the introduction of a carboxyl radical, and if the OH of this radical is replaced by an amido group, only slight changes in the pharmacological activity occur. The second group involves the ethyl esterization of the acid and the addition of a diethyl radical to the amido group. This produces a new pharmacologic action of a predominantly inhibitory type, the mechanism of which we have not been able to explain.

SUMMARY

A study has been made of the action of pyridine and some of its derivatives on the quadriceps and soleus muscles of cats anesthetized with nembutal. These substances are without effect on the activity of serum cholinesterase.

Pyridine (P). At lower frequencies of stimulation, this drug is without effect on the indirectly stimulated muscle. It has a brief depressant effect on directly stimulated muscle. As the frequency is increased, an enhancement of the contraction is usually obtained; occasionally it depresses. With higher frequencies, it produces a brief depression, followed by a more prolonged reinforcement. It antagonises curare, and reinforces slightly the effect of prostigmine. The response of the muscle to acetylcholine is enhanced. It does not modify the effect of acetylcholine and potassium injected during indirect stimulation.

Pyridine-3-carboxylic acid (nicotin, N). This drug is without effect on muscular contraction at low frequencies. At higher frequencies, it increases the tension; this is followed by a depression. It is antagonistic to curare and prostigmine. It is without effect on directly stimulated muscle. The response to acetylcholine is enhanced. It does not modify the effect of acetylcholine and potassium injected during indirect stimulation.

Pyridine-3-carboxylic acid amide (nicotinamide, NA). This drug does not have any effect on contraction produced by indirect stimulation at the frequen-

cies tested. It is antagonistic to curare. It increases the response to acetylcholine.

Ethyl ester of pyridine-3-carboxylic acid (EE). This substance decreases the tension developed during indirect stimulation at the frequencies studied. It increases the effects of curare and prostigmine. It produces a slight fall in the tension of directly stimulated muscle. It enhances the response of the muscle to acetylcholine; it increases the effects of acetylcholine and potassium injected during indirect stimulation.

Monoethyl-amide of pyridine-3-carboxylic acid (MA). This drug decreases the tension only at high frequencies of stimulation. It reinforces the action of curare and prostigmine.

Diethylamide of pyridine-3-carboxylic acid (Nikethamide, DA). This drug decreases the tension at high frequencies of stimulation. The effects of potassium and acetylcholine injected during indirect stimulation are enhanced.

The action of these drugs is discussed in relation to their chemical structure and may be interpreted in the light of their effects upon the threshold to acetylcholine.

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THE EFFECTS OF IODIDES, L-THIOSORBITOL, AND TWENTY-FIVE OTHER COMPOUNDS ON ALPHANAPHTHYLTHIOUREA (ANTU) TOXICITY IN RATS¹

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The mechanism of action of alphanaphthylthiourea (ANTU) in causing acute pulmonary edema and massive pleural effusion in susceptible animals without demonstrably affecting extra-pulmonary tissues has not yet been elucidated. Early reports, subsequently confirmed, on the toxicity of ANTU and related compounds to rats emphasized the striking differences in susceptibility between species and within species, apparently correlated with differences in age, strain, and diet (1-6). These variations have been extensively investigated by Richter and his coworkers (2, 4) who reported that strain differences could be equalized when all animals were put on a similar diet for a sufficient period of time, that carnivores were more susceptible than herbivores, and that the suckling wild Norway rat was seven times as resistant to ANTU as the adult, this resistance decreasing sharply around the time of puberty. Recent studies involving the use of rats castrated at an early age and suckling rats given daily doses of sex hormones indicated that the onset of sexual maturity coincided with the decrease in resistance to ANTU, but did not produce it (5).

Landgrebe (6) has reported that rats maintained on an oatmeal diet developed a greatly increased susceptibility to thiourea and that the additions of small quantities of iodide, bromide, calcium, desiccated thyroid, or methionine failed to protect these animals. However, tolerance was developed in these rats just as rapidly by repeated doses of the drug as in control animals.

In 1944, Griesbach *et al.* (7) discovered that daily subcutaneous injections of potassium iodide administered prophylactically protected their rats against otherwise lethal doses of thiourea, while potassium bromide or potassium chloride had no effect. Later, Byerrum (8) demonstrated that either Lugol's solution or potassium iodide supplied in the diet or drinking water for a period of one or two days prior to the injection of ANTU protected rats against several lethal doses of that compound, the degree of protection varying with the amount of iodide ingested.

In 1945, McClosky and Smith (9) found that, whereas reduction of dietary protein to a 4% level increased the susceptibility of rats to ANTU, the addition of 2% cystine to this diet restored the mortality rate to that of control animals on an adequate diet. In the same year, Richter (2) reported that feeding cystine

¹ A preliminary report of these studies appeared in *Federation Proceedings*, 6: 358 1947.

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to rats prevented the cessation of hair growth caused by chronic ingestion of ANTU. Shortly afterward, it was reported by DuBois *et al.* (10, 11) that cysteine, in a dose of 1 gram/kg injected intraperitoneally, completely protected rats against their "LD₁₀₀" dose of ANTU provided that the cysteine was administered within four hours after the ANTU injection.

Following the inability of workers in our laboratory to decrease the mortality in ANTU treated rats of the Wistar strain by means of large doses of parenterally administered cysteine, it was decided to investigate further the mechanism of action of ANTU.

MATERIAL AND METHODS. Albino, Wistar rats of both sexes,⁴ weighing from 200 to 300 grams, were maintained for no less than one month before use on a diet of Gaines dog biscuits, carrots, oranges, apples, cabbage, meat, peanuts, and bread dipped in milk or in cod liver oil.

Because of changes in the susceptibility of rats to thiourea derivatives with variations in strain and diet as reported elsewhere (1-5) and also noted in this laboratory, median lethal doses were determined by the method of Bliss (12) for all new shipments of animals and periodically for the stock colony. We have found that the LD₅₀ of ANTU for newly arrived rats may decrease as much as 100% during the first four weeks under our laboratory conditions. In the course of this series of experiments, the LD₅₀ varied from 2.5 to 5.9 mg./kg.

Throughout these experiments, injections of ANTU were given by the intraperitoneal route with constant, non-toxic quantities (1 cc./kg.) of redistilled propylene glycol as the vehicle. Substances administered prophylactically or therapeutically were given either orally or by injection, the intraperitoneal and intramuscular routes being used almost exclusively. Following studies on the rate of formation and disappearance of edema and effusion fluid, as reported below, the observation period for the experimental and control groups was set at 5 days.

Effect of position. Since the normal posture of the rat is such that gravitational forces might play a part in the formation of pulmonary edema and pleural transudate in such animals treated with thiourea or its derivatives, the effect of position was investigated. A group of 27 rats received approximately an LD₁₅ dose of ANTU intraperitoneally. Immediately after injection, 12 of these animals were placed in upright cylindrical containers made of $\frac{1}{2}$ " by $\frac{1}{2}$ " mesh screening, 15" in length and of such diameter as to keep the animals in an upright position and yet allow free movement along the length of the cylinders. Feed and water were placed so that they were accessible at all times. An additional 3 animals were placed head down in such containers. The remaining 12 rats were used as controls. The experimental animals were kept in the cylinders for seven hours, after which time the survivors were removed and placed in cages for further observation.

Rate and extent of edema and effusion formation in ANTU and ANTU-plus-cysteine treated animals. In order to determine the rate and extent of pulmonary edema and pleural transudate formation in response to various doses of ANTU, and the effect of concomitantly administered cysteine on such formation, a total of 82 rats was sacrificed at progressive intervals after the injection of either $\frac{1}{2}$, 1, or 2 LD₅₀'s of ANTU corresponding to expected mortalities of 5%, 50%, and 95%, respectively. The symptoms at the time of sacrifice, the amount and character of the pleural effusion and peritoneal transudate, and the gross appearance of the lungs were noted. Thirty-seven of these animals received intraperitoneal or intramuscular injections of cysteine, 1 gram/kg., simultaneously with the ANTU. There were, in addition, 6 animals that received the same amount of cysteine given intra-

⁴ We have noted no sex difference in susceptibility. This is in accord with the work of Richter (4).

peritoneally alone and 4 animals that received propylene glycol, 1 cc./kg., intraperitoneally alone.

The prophylactic and therapeutic effectiveness of various compounds against the acute toxicity of ANTU. The prophylactic and therapeutic effectiveness of twenty-five compounds against the acute toxicity of ANTU was studied in the following manner: Water soluble compounds were prepared in aqueous solution and neutralized if necessary, while insoluble material was suspended in an aqueous 1% solution of sodium methyl carboxylic cellulose or dissolved in propylene glycol. The dose and volume of the substances injected were adjusted to the body weight of the animal. In all instances appropriate controls were run to eliminate the possibility of drug or vehicle toxicity. Compounds were usually given in maximal sub-lethal dosage. Tests were conducted, with few exceptions, on groups of ten animals. Injections of ANTU were generally limited to not more than two intraperitoneal LD₅₀'s, equivalent to an expected mortality of about 90-95%. On each test-day, one group of ten animals injected with freshly prepared ANTU served as the control group for that day. The compounds tested and the rationale behind their selection are given below.

(a) Potassium iodide was studied in order to confirm and extend, if possible, information already in the literature (7, 8) concerning the protective action of this substance when administered prophylactically. Inasmuch as inorganic iodide is effective only when given prophylactically, it is likely that incorporation of the iodide into an organic compound *in vivo* is necessary before it can exert its protective effect. For this reason the organic iodides diiodotyrosine, cetyl iodide,⁴ n-decyl iodide,⁵ amyl iodide, and iodoacetic acid were tested. The latter compound was of special interest because of its reported reaction with —SH groups (13, 14). The use of iodine was suggested by the work of DeRobertis and Grasso (15) who reported that cellular peroxidases normally catalyzing the liberation of iodine from iodide in thyroid tissue were inhibited by the thiourea compounds, and by the work of DuBois and Erway (16) who found that the inhibition of tyrosinase by ANTU could be prevented completely by the addition of iodine to the enzyme at the same time, while potassium iodide in high concentrations had no effect on this inhibition.

(b) On the basis of the literature (10, 11) relative to the efficacy of therapeutically administered cysteine in preventing completely the symptoms and signs of ANTU intoxication in Sprague-Dawley and in Wilson rats, and in view of the possibility of a disulfide linkage (17) or molecular compound formation between ANTU and —SH compounds, cysteine and three other —SH containing substances, 2,3-dimercaptopropanol (BAL), BAL glucoside and l-thiosorbitol,⁶ a sugar previously found to be effective by Harvey *et al.* (18) in these laboratories, were investigated.

(c) The reported effectiveness of the —SH compounds suggested the use of labile sulfur-containing substances and of compounds with easily oxidizable groups such as —CHO. Therefore, sodium thiosulfate, diphenyl sulfide⁷ and diphenyl disulfide,⁷ as well as n-heptaldehyde and dextrose were used. Regarding the latter, it should be noted that hyperglycemia following ANTU poisoning has been reported (11, 19).

(d) Because of the possibility of an *in vivo* chemical reaction between the amino group of ANTU and a carboxyl group, the aliphatic amino acid glycine and the aromatic acids paraminobenzoic acid and nicotinic acid were included among the compounds to be tested.

(e) Since the acute toxicity of ANTU is probably due to some action which produces a markedly increased permeability of pulmonary capillaries, and since histamine is a vasodilator which in sufficient dosage causes increased capillary permeability, it seemed possible that ANTU causes a local liberation of a histamine-like substance. Consequently the effects of two anti-histamine drugs, benadryl⁸ and pyribenzamine,⁹ were evaluated.

⁴ Supplied through the courtesy of the Columbia Organic Chemicals Company.

⁵ Supplied through the courtesy of the Dupont Experimental Station, Wilmington.

⁷ Supplied through the courtesy of the General Chemical Company.

⁸ Supplied through the courtesy of Parke, Davis and Company.

⁹ Supplied through the courtesy of Ciba Pharmaceutical Products.

(f) To determine how drug-induced hypertension and stimulation of the respiratory center would affect ANTU-treated animals, ephedrine sulfate and coramine were used.

(g) The selection of tyrosine was based on the reports of interference by thiourea derivatives in pigment formation (2, 20, 21) and their inhibition of tyrosinase *in vitro* (16, 22, 23).

(g) Since hexamethylenetetramine had been proven effective prophylactically in preventing the pulmonary edema produced by inhalation of phosgene (24), its use was hazarded on the possibility that in addition to a direct combination with phosgene it also acted by decreasing capillary permeability.

(i) Finally, because l-thiosorbitol proved to be more effective than other —SH compounds, sorbitol alone was tried.

RESULTS. *Effect of position.* There was no significant difference in time of onset of symptoms, time of death, or in total mortality between the control group and the groups of 12 and 3 animals kept, respectively, in an upright and in a head-down position for seven hours following the injection of ANTU.

Rate and extent of edema and effusion formation in ANTU and ANTU-plus-cysteine treated animals. The results of this experiment are summarized in table 1. It was found that measurable amounts of pleural transudate may appear within 3 hours following an injection of $\frac{1}{2}$ LD₅₀ of ANTU. The effusion fluid was clear, straw-colored, and usually clotted within 15 minutes of removal from the thoracic cage. Other workers (1, 2, 25, 26, 27) have shown that this fluid has a high protein content very similar to that of lymph and rapidly forms an extensive fibrin network. It should be noted that in nearly every group there were marked variations in symptomatology and gross pathological findings. Throughout this experiment it was frequently found that animals had an appreciable amount of pleural transudate without symptoms or gross evidence of pulmonary edema, and it seems logical to assume that the degree of dyspnea, during the first few hours at least, is directly dependent upon the extent of pulmonary edema rather than upon the amount of pleural effusion present. Rats dying within the first six hours often had massive pulmonary edema with comparatively small amounts of pleural transudate.

Animals receiving simultaneous injections of ANTU and cysteine showed qualitatively the same findings as noted for those receiving ANTU alone, but there was a definite delay in the onset of symptoms and in the accumulation of effusion and edema fluid in the cysteine treated rats. Also, while the animals receiving ANTU alone had either small amounts of clear, straw-colored, ascitic fluid shortly after injection or none at all, those receiving cysteine intraperitoneally had, even though the latter was neutralized with NaOH immediately prior to injection, comparatively large volumes (1–10 cc.) of cloudy, straw-colored, occasionally serosanguinous, ascites which clotted shortly after removal from the peritoneal cavity. Because of the large quantity of fluid found in the peritoneal cavities of the cysteine treated rats, it was considered possible that the delay in the onset of symptoms and pathological findings in these was due to the diversion into the peritoneal cavity of fluid that otherwise might have found its way into the lungs. Consequently, a group of rats was given the cysteine intramuscularly rather than intraperitoneally, and, in spite of the fact that ascites was not produced in these animals, the delay in symptoms and in accumu-

lation of pulmonary effusion and edema fluid was again definitely shown. However, in these, as in our other experiments, it was apparent that this delay in symptoms was temporary and that the mortality was not significantly decreased

TABLE 1

The rate and extent of pulmonary edema and effusion formation following injection of ANTU, and ANTU plus cysteine

DRUG	ROUTE	NUMBER LD50'S (ANTU)	NUMBER ANI- MALS	SACRI- FICED (HRS. AP- TER IN- JECTION)	SYMPTOMS* (DYSPNEA)	PULMONARY EDEMA	PLEURAL EFFUSION†	ASCITES
							cc.	cc.
ANTU	IP	½	3	3	0	0	1 (0-2)	1 (0-3)
ANTU	IP	½	3	6	0	0	3 (2-5)	0
ANTU	IP	½	3	2½	0	0	0	0
ANTU	IP	1	4	3	0-+++	?-+++	2½ (2-3)	1½ (1-3)
ANTU	IP	1	4	6	+ -+++	?-+++	4½ (3-7)	?
ANTU	IP	1	7	2½	0-+++	?-+++	6 (1-12)	0
ANTU	IP	1	4	48	0	0-?	1 (0-3)	0
ANTU	IP	1	2	72	0	0	0	0
ANTU	IP	2½	6	3	+ -+++	+ -+++	4 (3-6)	1 (2-3)
ANTU	IP	2	6	6	+ -+++	+ -+++	5 (2-8)	½ (0-2)
ANTU	IP	2	1	2½	++	++	7½	0
ANTU	IP	1	4	3	0	0	0	6 (5-8)
Cysteine ‡	IP	1	4	6	0-+	0-?	1 (0-2)	5 (1-10)
ANTU	IP	1	4	6	0-+	0-?	1 (0-2)	5 (1-10)
Cysteine	IP	1	4	2½	+ -+++	+ -+++	5 (2-9)	2 (1-3)
ANTU	IP	2	5	3	0	0	½ (0-2)	7 (5-8)
Cysteine	IP	2	3	6	0-++	?-+	1½ (1-3)	4½ (4-6)
ANTU	IP	2	2	24	+ -+++	+ -+++	6 (3-9)	0
Cysteine	IP	2	5	3	0	0	1 (0-3)	1 (0-2)
ANTU	IP	2	2	24	+ -+++	+ -+++	5½ (4-7)	0
Cysteine	IM							

* 0 = none; ? = questionable; + = slight; ++ = moderate; +++ = marked.

† Usually clear, straw-colored and clotted within 15 minutes of removal by means of a fine-tipped pipette.

‡ Equivalent to an expected mortality of ca. 5%.

§ Equivalent to an expected mortality of ca. 90%.

¶ Cysteine HCl neutralized with NaOH and a dose of 1 gm./kg. injected simultaneously with the ANTU.

by the administration of cysteine. This was, as will be shown later, in contrast to the results with l-thiosorbitol. With few exceptions, throughout all of the experiments, the animals given either ANTU alone or ANTU with cysteine,

regardless of the route by which the latter was administered or of the time of administration, died during the period between 4 and 18 hours after the injection of ANTU.

Intraperitoneal injections of cysteine alone produced no pleural effusion or lung changes but produced ascites similar in character and amount to that obtained when cysteine was injected with ANTU.

Intraperitoneal injections of propylene glycol alone (1 cc./kg.) produced no effusion or lung changes and only slight, fleeting ascites.

The prophylactic and therapeutic effectiveness of various compounds against the acute toxicity of ANTU. The complete protocols for these experiments appear in tables 2-6. In addition, the structural formulae and molecular weights of all compounds used are presented in table 7. Of the twenty-five compounds tested, only potassium iodide (table 2) and l-thiosorbitol (table 3) were unequivocally successful in decreasing the mortality produced by ANTU. Thiosorbitol was effective therapeutically; potassium iodide, only prophylactically. Cysteine, as mentioned previously, caused a definite delay in onset of symptoms but, under the conditions of a large series of experiments, produced no significant decrease in mortality (table 4). Negative results, in several cases suggesting increased susceptibility to ANTU, were obtained with the other compounds investigated (table 5). Ephedrine, for example, definitely increased the susceptibility of the rats to ANTU, a finding which was expected as it seemed probable that increased filtration pressure in the presence of an increased capillary permeability would result in earlier and more extensive capillary leakage.

Potassium iodide afforded no protection against the toxic effects of ANTU when the former was given either intraperitoneally or intravenously, in maximal sub-lethal dosage, simultaneously with the poison (table 2). Similar results were obtained when the iodide was administered, by either the intraperitoneal or subcutaneous route, in a dose of 250 mg./kg. six hours before the injection of ANTU. However, when this amount was injected twenty-four hours prior to the administration of the poison, the expected mortality was reduced from 95% to 50%. When the period between iodide and ANTU injections was extended to forty-eight hours, the mortality was reduced to zero, nearly all animals of the experimental group remaining symptomless. On the other hand, reducing the dose of iodide to 100 mg./kg. similarly administered resulted in no protection at all. The administration of the 250 mg./kg. dose in the drinking water or by repeated subcutaneous injections during the forty-eight hour period produced a definite reduction in mortality to 10% and 35% respectively, but did not give the complete protection furnished by the same amount of iodide injected in one dose forty-eight hours before the administration of ANTU. When the period between iodide and rodenticide injections was increased to seventy-two hours, a 5% mortality was obtained, with approximately half the survivors showing mild dyspnea. A delay of 4 days and 5 days resulted in mortalities of 30% and 40%, respectively, while a delay of six days resulted in a mortality of 45%. Rats injected with ANTU 10 days after the administration of the iodide no longer received any protection at all. These results are graphically presented in fig. 1.

The five organic iodides and potassium bromide gave neither therapeutic nor prophylactic protection (table 5).

TABLE 2
*The effect of potassium iodide on ANTU toxicity**

TIME OF ADMINISTRATION†	DOSE‡	ROUTE	CONTROL MORTALITY	EXPERIMENTAL MORTALITY
	mg./kg.			
Simultaneously.....	250	IP	10/10	9/10
Simultaneously.....	500	IP	8/10	9/10
Simultaneously.....	25	IV	10/10	10/10
6 hr. before.....	250	IP	19/20	18/20
24 hr. before.....	250	IP	9/10	4/10 (✓)
48 hr. before.....	250	IP	8/10	0/30 (✓)
48 hr. before.....	100	IP	9/10	10/10
48 hr. before.....	300±	Drinking water§	9/10	1/10 (✓)
48 hr. before.....	250	IP, SC¶	10/10	7/20 (✓)
72 hr. before.....	250	IP	19/20	1/20 (✓)
4 days before.....	250	IP	8/10	3/10 (✓)
5 days before.....	250	IP	9/10	8/20
6 days before.....	250	IP	9/10	9/20
10 days before.....	250	IP	10/10	19/20

* 2 LD₅₀'s of ANTU, equivalent to an expected mortality of ca. 95%.

† In relation to ANTU injection.

‡ Aqueous solution. (50 mg./cc.).

§ 2 mg./cc., given for a period of 48 hr. before ANTU injection.

¶ Divided into four equal doses and injected twice daily for two days.

(✓) $P < 0.01$ by Student's "t" Test.

TABLE 3
The effect of 1-thiosorbitol on ANTU toxicity*

ROUTE	TIME OF ADMINISTRATION†	NUMBER LD ₅₀ 's (ANTU)	CONTROL MORTALITY	EXPERIMENTAL MORTALITY‡
IP, IM	Simultaneously	2½	27/30	14/30 (✓)
IM	½ Simultaneously			
	½ 3 hrs. after	2	8/10	3/10 (✓)
IP	Simultaneously	2½	10/10	9/10

* 1½ gm./kg., in aqueous solution (150 mg./cc.).

† In relation to ANTU injection.

‡ Animals dying showed a delay in onset of symptoms and time of death.

§ Equivalent to an expected mortality of ca. 95%.

(✓) $P < 0.01$ by Student's "t" Test.

The addition of an iodine-potassium iodide solution to the ANTU solution in a proportion of five moles of free iodine to one mole of ANTU immediately prior to injection completely destroyed the toxicity of the rodenticide (table 6), the fifteen animals injected remaining symptomless. Similar results were obtained

with a bromine-potassium bromide solution, iodoacetic acid, and sodium iodoacetate, while the addition of potassium iodide or potassium bromide alone had no effect on the toxicity of the ANTU. However, while iodine, iodoacetic acid, and sodium iodoacetate were highly effective in blocking the toxic action of ANTU by *in vitro* mixing, they were found to be completely ineffective *in vivo*, maximal sublethal doses given intravenously simultaneously with or in divided doses after the administration of the rodenticide producing no decrease in mortality (table 5). Many of the animals receiving high doses of free iodine intravenously developed a hemorrhagic diathesis manifested by epistaxis, hematuria, and the appearance of many RBC's in their pleural effusions. Their response to ANTU, however, was no different from that of the controls.

Because these animals were injected via the femoral vein under nembutal (sodium pentobarbital) anesthesia, and because of the reported efficacy of hypnotics in the treatment of the experimentally produced "neurogenic" type of

TABLE 4
The effect of cysteine on ANTU toxicity*

ROUTE	TIME OF ADMINISTRATION†	NUMBER LD ₅₀ 'S (ANTU)	CONTROL MORTALITY	EXPERIMENTAL MORTALITY‡
IP, IM	4 hrs. before	25	17/20	16/20 ~
IP	Simultaneously	1	5/10	13/20
IP, IM, SC	Simultaneously	2	33/35	37/45
IP, IM	‡ Simultaneously	2	8/10	18/20
	‡ 3 hrs. after			

* Cysteine HCl, 1 gm./kg., in aqueous solution (100 mg./cc.) neutralized with NaOH and used within ½ hour of preparation.

† In relation to ANTU injection.

‡ Animals dying showed a delay in onset of symptoms and, frequently, in time of death.

§ Equivalent to an expected mortality of ca. 90%.

pulmonary edema (28), a group of rats was given only nembutal and ANTU. It was found that sustained nembutal anesthesia had no effect on the response of rats to ANTU, symptoms appearing and death occurring at approximately the same time as in unanesthetized controls (table 5).

Thiosorbitol consistently reduced mortality when given in a dose of 1.5 gm./kg., intraperitoneally or intramuscularly, simultaneously with the administration of 2 LD₅₀'s of ANTU (table 3). When the amount of ANTU was increased to 2.5 LD₅₀'s, thiosorbitol did not decrease the mortality; however, the time of onset of symptoms and of death was delayed in this as in the other thiosorbitol treated groups. Because of our limited supply of this compound, we were unable to determine the effect of varying the dose or of administering the drug at intervals after the injection of ANTU.

DISCUSSION. The problems involved in attempting to elucidate the mechanism or mechanisms of action of ANTU and related compounds are many and complex. The peculiar distribution of the pathology caused by these compounds

TABLE 5
Compounds ineffective against ANTU

COMPOUND	DOSE	PREPARATION	ROUTE OF ADMINISTRATION	TIME OF ADMINISTRATION (REFERENCE TO ANTU)	NUMBER LD ₅₀ 'S (ANTU)	BODY WEIGHT (AVERAGE)	CON. TROL MORTALITY	EXPERI. MORTALITY
	mg/kg							
BAL	25	Propylene glycol solution	IM	3 hr before, 3 simult	2	245	7/10	9/10
BAL	25	Propylene glycol solution	IP	Simultaneously	1	253	4/10	8/10
BAL	25	Propylene glycol solution	IM	3 simult, 3 hr after	2	229	9/10	9/10
BAL glucoside	100	1 M sulfate sol.†	IP	Simultaneously	2	243	10/10	9/10
Sodium thiosulfate	500	Aqueous sol	IP	Simultaneously	2	225	10/10	10/10
Sodium thiosulfate	500	Aqueous sol	IM	3 simult, 3 hr after	2	225	8/10	9/10
Diphenyl sulfide	550	Undiluted	IP	Simultaneously	2	250	9/10	10/10
Diphenyl disulfide	200	1% cellulose‡	IP	Simultaneously	2	255	9/10	5/5
Dextrose	20 000±	Aqueous sol	Drinking water§	Period of 72 hr before	2	229	8/10	10/10
Dextrose	2 000	Aqueous sol	IM	Simultaneously	2	254	8/10	10/10
n Heptaldehyde	1,700	Undiluted	IM	4 hr before	2	252	9/10	10/10
n Heptaldehyde	1,700	Undiluted	IM	Simultaneously	2	264	9/10	9/10
Glycine	500	Aqueous solution	IP	Simultaneously	2	227	9/10	9/10
Glycine	500	Aqueous solution	IM	3 simult, 3 hr after	2	219	9/10	10/10
Paraminobenzoic acid	500	Aqueous solution†	IP	Simultaneously	1	246	5/10	5/10
Nicotinic acid	500	Aqueous solution	IP	Simultaneously	2	230	10/10	8/10
Nicotinic acid	500	Aqueous solution	IM	3 simult, 3 hr after	2	228	10/10	9/10
Benadryl	25	0.9% saline	IP	Simultaneously	1	246	5/10	8/10
Pyribenzamine	25	0.9% saline	IP	Simultaneously	1	251	5/10	7/10
Ephedrine sulfate	100	Aqueous solution	IM	Simultaneously	1	218	4/10	10/10
Coramine	50	Aqueous solution	IM	Simultaneously	1	224	4/10	4/10
Coramine	100	Aqueous solution	IM	3 simult, 3 hr after	1	227	4/10	5/10
Tyrosine	500	1% cellulose‡	IM	45 hr, 24 hr before	2	241	8/10	5/5
Tyrosine	250	1% cellulose	IP	Simultaneously	2	245	8/10	9/10
Hexamethylene-tetramine	1,000	Aqueous solution	IM	4 hr before	2	264	8/10	9/10
Hexamethylene-tetramine	1,000	Aqueous solution	IM	Simultaneously	2	238	8/10	10/10
Sorbitol	1,500	Aqueous solution	IM	4 hr before	2	234	9/10	8/10
Sorbitol	1 500	Aqueous solution	IP, IM	Simultaneously	2	245	19/20	15/20
Sorbitol	1 500	Aqueous solution	IM	3 simult, 3 hr after	2	255	9/10	16/20
Dnodo'tyrosine	500	1% cellulose‡	IP	45 hr before	2	242	9/10	5/5
Duodotyrosine	500	1% cellulose	IP	Simultaneously	2	251	9/10	8/10

* LD₅₀s varied from 2.5 to 5.9 mg/kg during the course of these experiments. 2 LD₅₀s were equivalent to an expected mortality varying from 60 to 95%.

† Neutralized with NaOH before use.

‡ Aqueous solution of sodium methyl carboxylic cellulose.

§ Given as 10% dextrose in drinking water for 72 hrs prior to injection of ANTU.

TABLE 5—Continued

COMPOUND	DOSE	PREPARATION	ROUTE OF ADMINISTRATION	TIME OF ADMINISTRATION (REFERENCE TO ANTU)	NUMBER LD50'S* (ANTU)	BODY WEIGHT (AVERAGE)	CONTROL MORTALITY	EXPERIMENTAL MORTALITY
	mg/kg							
Cetyl iodide	560	Undiluted	IP	48 hr before	2	238	9/10	5/5
Cetyl iodide	560	Undiluted	IP	Simultaneously	2	257	9/10	10/10
n Decyl iodide	415	Undiluted	IP	48 hr before	2	253	9/10	5/5
n Decyl iodide	415	Undiluted	IP	Simultaneously	2	248	9/10	9/10
Amyl iodide	300	Undiluted	IP	Simultaneously	2	260	10/10	10/10
Iodoacetic acid	25	Aqueous solution ‡	IM	48 hr before	2	258	10/10	9/10
Iodoacetic acid	25	Aqueous solution	IP	Simultaneously	2	257	10/10	10/10
Iodoacetic acid	25	Aqueous solution	IV	15 minutes after	2	231	10/10	9/10
Sodium iodoacetate	25	Aqueous solution	IV	15 minutes after	2	238	10/10	10/10
Iodine—potassium iodide	25-48	Aqueous solution	IV	15 minutes after	2	252	8/10	5/5
Iodine—potassium iodide	25-48	Aqueous solution	IV	‡ 15 min, ‡ 3 hr after	2	243	8/10	5/5
Potassium bromide	400±	Aqueous solution	Drinking water	Period of 48 hr before	2	227	9/10	5/5
Sodium pentobarbital	80	Aqueous solution	IP	‡ 15 min, ‡ 2 hr after	2	245	8/10	9/10

* Used within ‡ hr of preparation

TABLE 6

The effect of addition of halogen compounds to ANTU solution* prior to injection

COMPOUND†	DOSE	CONTROL MORTALITY	EXPERIMENTAL MORTALITY
	mg/kg		
I-KI	25-48	12/15	0/15
Br-KBr	32-ca 75		0/5
Iodoacetic acid	25		0/5
Na iodoacetate	25		0/5
KI	84‡		5/5
KBr	100		4/5

* 2 LD50's, equivalent to an expected mortality of ca 95%

† All prepared as aqueous solutions and added to the ANTU solution (1 part to 4) immediately prior to injection

‡ I content equivalent to total amount of I in above I-KI solution

(1, 2, 4, 25, 26), the variation in susceptibility with differences in species, strain, age, and diet (1-6), the rapid appearance of tolerance (2, 6, 9, 20, 29), and the effect on pigmentation and hair growth (2, 20, 21) have been discussed elsewhere.

The protective effect of iodide administered prophylactically to animals subsequently receiving an otherwise lethal dose of ANTU was confirmed. Iodide administered simultaneously with the rodenticide, even though the former was given intravenously, gave no protection at all, and our experiments indicate

TABLE 7

Structural formulae and molecular weights of compounds used in this study

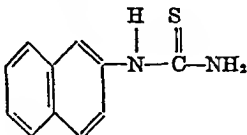
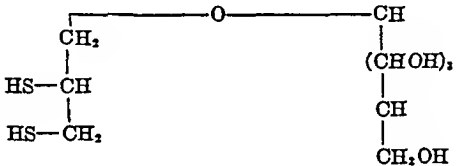
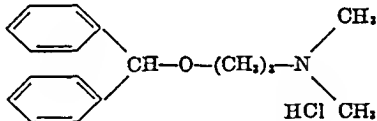
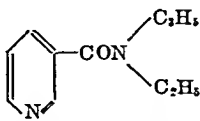
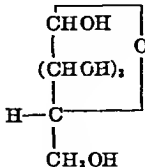
COMPOUND	FORMULA	MOLECULAR WEIGHT
n-Amyl Iodide	$\text{CH}_3-(\text{CH}_2)_4-\text{CH}_2\text{I}$	198.06
ANTU		202.27
BAL	$\begin{array}{c} \text{HS}-\text{CH}_2-\text{CH}-\text{CH}_2\text{OH} \\ \\ \text{SH} \end{array}$	124.21
BAL glucoside		286.35
Benadryl		291.81
n-Cetyl iodide	$\text{CH}_3-(\text{CH}_2)_{14}-\text{CH}_2\text{I}$	352.34
Coramine		178.23
Cysteine	$\begin{array}{c} \text{HOOC}-\text{CH}-\text{CH}_2\text{SH} \\ \\ \text{NH}_2 \end{array}$	121.15
n-Decyl iodide	$\text{CH}_3-(\text{CH}_2)_9-\text{CH}_2\text{I}$	268.19
Dextrose		180.16

TABLE 7—Continued

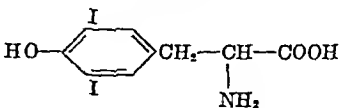
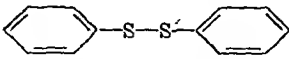
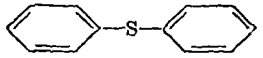
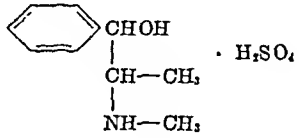
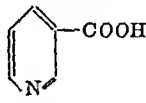

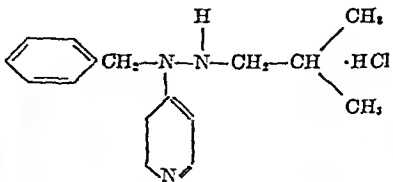
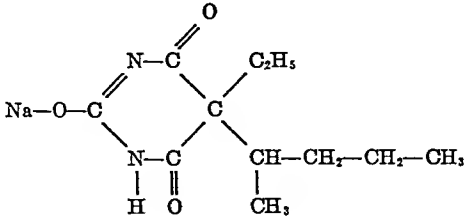
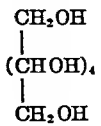
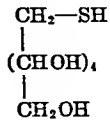
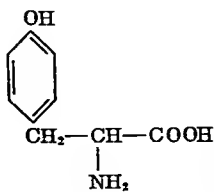
COMPOUND	FORMULA	MOLECULAR WEIGHT
Diiodotyrosine		433.01
Diphenyl disulfide		218.32
Diphenyl sulfide		186.26
Ephedrine sulfate		263.30
Glycine	$\text{NH}_2\text{—CH}_2\text{—COOH}$	75.07
n-Heptaldehyde	$\text{CH}_3\text{—(CH}_2)_5\text{—CHO}$	114.18
Hexamethylenetetramine	$(\text{CH}_2)_6\text{N}_4$	140.19
Iodine	I_2	253.84
Iodoacetic acid	$\text{I—CH}_2\text{COOH}$	185.96
Nicotinic acid		123.12
PABA		137.13
Potassium bromide	KBr	119.01
Potassium iodide	KI	166.02
Pyribenzamine		291.82

TABLE 7—Continued

COMPOUND	FORMULA	MOLECULAR WEIGHT
Sodium pentobarbital		248.26
Sodium thiosulfate	$\text{Na}_2\text{S}_2\text{O}_3$	158.11
Sorbitol		182.17
l-Thiosorbitol		198.23
Tyrosine		181.19

that the iodide must be administered more than six hours prior to the injection of ANTU in order to exert some protective action. These results suggest that following the administration of potassium iodide a definite period of time is necessary either for the formation of an organic iodide which is then able to interact with ANTU to produce a non-toxic substance, or, possibly, for the conversion of iodide to an excess of free iodine, the latter then reacting with the thiourea present and rendering it incapable, perhaps by oxidation to the disulfide as suggested by the work of Mackenzie (30) and Miller *et al.* (31), of interfering with one or more steps in oxidative enzyme processes. While the latter possibility is given credence by the previously mentioned reports of DeRobertis and Grasso (15) and DuBois and Erway (16) as well as by our findings that free iodine is capable of reacting with ANTU *in vitro* and completely destroying its toxicity, the fact that we were unable to obtain protection *in vivo* by intra-

venously administered iodine appears to be evidence against it. Studies in other laboratories with radioactive iodine have shown that it is quickly brought to the thyroid gland, the principal storehouse of the body for this element, and rapidly converted by the thyroid cells into organic compounds which in a matter of a few hours may return to the circulation. It is interesting to note that, second to the thyroid gland, the lungs were shown to have collected most of the iodine injected (32, 33). Since none of the organic iodides that we tested in the present study are highly reactive, it is our intention to investigate less stable organic iodides.

The difference in the degree of protection against ANTU offered by cysteine in this laboratory as compared with that found by DuBois *et al.* (10, 11) cannot be accounted for except, perhaps, on the basis of strain and/or dietary variation.

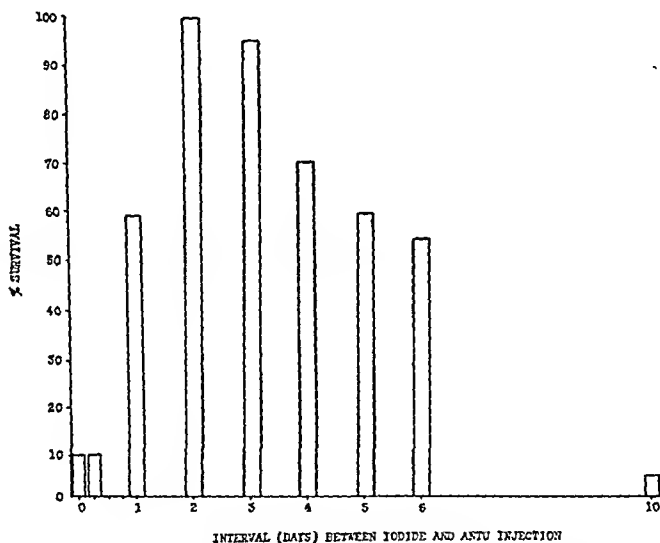


FIG. 1. EFFECT OF A PROPHYLACTIC 250 MG./KGM. DOSE OF POTASSIUM IODIDE ON ANTU TOXICITY

Two different lots of cysteine were utilized in our experiments. If, as suggested by the work of Toennies (17), thiourea derivatives combine with —SH compounds in the body to form disulfides, and cysteine exerts a protective action either by tying up the poison before it can act upon such tissue constituents as glutathione, methionine, or one of the —SH containing enzymes, or by competing with ANTU for such constituents, one might assume that another monothiol, l-thiosorbitol, would confer an equivalent amount of protection. Our results indicate, however, that, even on a molar basis, thiosorbitol furnishes significantly greater protection than does cysteine. Although we found the dithiols, BAL and BAL glucoside, to be ineffective in protecting our rats against ANTU, it should be emphasized that because of its toxicity BAL cannot be given in an amount larger than 25 mg./kg., equivalent on a molar basis to approximately $\frac{1}{8}$ the dose

of thiosorbitol that was used. BAL glucoside is much less toxic, but because we had a limited amount of this very unstable substance, we were able to test only a 100 mg./kg. dose. Larger amounts may very well prove to be effective.

The results with sustained nembutal anesthesia suggest that there is no neurogenic factor involved in the development of the acute pulmonary edema shown by ANTU poisoned rats.

The unique characteristics of pulmonary tissue, as pointed out by Drinker (25), may serve, at least partially, to explain the apparently isolated pathology caused by the thiourea compounds. Recent work, however, has indicated that in the blood vessels of different organs, different chemical mechanisms may operate, giving rise to qualitative differences in response to various enzyme inhibitors (34). At any rate, the capillaries of the lung do possess some quality, either of position or of constitution, which makes them specifically vulnerable to these drugs, and it is equally certain that the pulmonary lymphatic drainage system of many mammals is such that it is not capable of draining off a real excess of pulmonary tissue fluid (25). Drinker refers to the right lymphatic duct as a "bottleneck" at the end of a widespread system of lymphatic vessels. It is conceivable that the degree of obstruction produced by this "bottleneck" varies with species and even with strains and age groups within the same strain and in this way might account for some of the variations in response to the thiourea compounds. This possibility is being currently investigated by this laboratory.

The depression of metabolism as indicated by the rapid and sustained fall in body temperature after the administration of ANTU (2), the rapid appearance of hyperglycemia along with an irreversible depletion of liver glycogen (10, 11, 19), the interference with melanin formation, and the ability of the thiourea compounds to suppress thyroid hormone production all seem to indicate that at least some of the effects produced by these compounds are due to an interference with one or more enzyme systems. Of the many specific enzymes that have been tested *in vitro* with thiourea compounds, only two, tyrosinase (16, 22, 23) and peroxidase (15), have been found to be inhibited by these compounds. Copper-protein enzymes such as tyrosinase have not been shown to play an important part in the physiology of the mammalian body, and it seems improbable that the toxic effects of the thioureas can be explained by their action on tyrosinase. The recent paper by DeRobertis and Grasso (15) indicates that thiourea has an inhibitory action on the peroxidase system of normal thyroid tissue and in this way prevents the liberation of iodine from iodides, the iodination of tyrosine to di-iodotyrosine and, consequently, the ultimate formation of thyroxine. Further investigation of the peroxidase activity in other tissues and of the effect of thiourea compounds on the oxidation of other physiologically important substrates by peroxidase may serve to clarify considerably the mechanism of action of these compounds.

SUMMARY

(1) Of twenty-five compounds tested only potassium iodide and l-thiosorbitol were unequivocally successful in preventing the lethal pulmonary edema and

pleural effusion caused by the administration of ANTU to albino rats of the Wistar strain. Cysteine caused a delay in the onset of symptoms and in the appearance of edema and effusion fluid but did not significantly decrease mortality. Negative results were obtained with other sulfhydryl and labile sulfur compounds (BAL, BAL glucoside, sodium thiosulfate, diphenyl sulfide, and diphenyl disulfide), reducing substances (dextrose and n-heptaldehyde), aliphatic and aromatic acids (glycine, paraminobenzoic acid, and nicotinic acid), anti-histamine drugs (benadryl and pyribenzamine), vasomotor and respiratory stimulants (ephedrine and coramine), a melanin precursor (tyrosine), a prophylactic against phosgene pulmonary edema (hexamethylenetetramine), a hexanehexol (sorbitol), organic iodides (diiodotyrosine, cetyl iodide, n-decyl iodide, amyl iodide, and iodoacetic acid) and iodine.

(2) Potassium iodide protected only when given prophylactically. Complete protection was obtained by the intraperitoneal injection of a single 250 mg./kg. dose 48 hours prior to the administration of 2 LD₅₀'s of ANTU. Decreasing or increasing this period between iodide and ANTU injection resulted in decreasing degrees of protection; animals receiving the iodide at six hours or ten days before the administration of the ANTU were afforded no protection at all. Reduction of the dose of iodide to 100 mg./kg. resulted in complete loss of protection, regardless of the time of administration. The 250 mg./kg. dose was more effective when administered by a single injection 48 hours before the ANTU than when given in divided doses or in drinking water during the 48 hour period.

(3) The addition of free iodine, bromine, iodoacetic acid, or sodium iodoacetate to the ANTU solution immediately prior to injection completely destroyed the toxicity of the rodenticide, while the addition of potassium iodide or potassium bromide alone had no effect. However, iodine, iodoacetic acid, and sodium iodoacetate were found to be ineffective *in vivo*.

(4) Thiosorbitol was effective therapeutically, consistently reducing mortality when given in a dose of 1.5 gm./kg. simultaneously with the administration of 2 LD₅₀'s of ANTU.

(5) Position was shown to have no influence on the response of rats to ANTU.

(6) Sustained nembutal anesthesia was shown to have no influence on the response of rats to ANTU, a finding which suggests that there is no neurogenic factor involved in the development of the acute pulmonary edema shown by ANTU poisoned rats.

(7) Data on the rate and extent of pulmonary edema and pleural transudate formation in response to various doses of ANTU, and on the effect of concomitantly administered cysteine on such formation, are presented.

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DETERMINATION OF THE THERAPEUTIC, IRREGULARITY, AND LETHAL DOSES OF CARDIAC GLYCOSIDES IN THE HEART-LUNG PREPARATION OF THE DOG¹

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Attempts to determine the therapeutic activity of a number of cardiac glycosides have resulted in contradictory results. Measuring oxygen consumption of the heart-lung preparation (HLP) at constant diastolic volume and calculating the efficiency of cardiac muscle, Visscher (1), Moe and Visscher (2), and Moe (3) have found marked differences in the ratio of minimal irregularity dose to minimal therapeutic dose with certain cardiac glycosides. Thus, with lanatoside C and g-strophanthin the ratio of irregularity dose to therapeutic dose was 1.8 and 2.3 respectively. Lanatoside A and B had ratios of 0.73 and 0.7 respectively (3). The latter ratios indicate the appearance of increased efficiency only after the appearance of cardiac irregularities with lanatosides A and B. Takahashi et al. (4), using the intact rabbit, claim that differences in the ratio of toxic dose to therapeutic dose exist for various tinctures of digitalis as well as for a number of crystalline glycosides. According to Cattell and Gold (5) and White and Salter (6), who have used the isolated papillary muscle of the cat, there are no significant differences in the ratio of therapeutic dose to toxic dose of lanatosides A, B and C and of a number of other cardiac glycosides. These contradictory results have made it desirable to use the HLP to restudy this question.

It has been previously shown (7) that the lethal dose (L.D.) of g-strophanthin and digitoxin in the HLP is dependent on the rate of administration of these glycosides. In this paper we have extended this observation to the glycosides digoxin, oleandrin, and lanatoside B. We also have investigated the influence of the rate of administration of the five glycosides upon the therapeutic dose and the irregularity dose.

MATERIALS AND METHODS. In the present study the crystalline glycosides digitoxin,³ g-strophanthin,⁴ digoxin,⁵ oleandrin,⁶ and lanatoside B,⁷ have been employed. Unless otherwise stated, all dosages given are on a molar basis. The molecular weights used were: g-strophanthin, 648.7; digitoxin, 764.5; digoxin, 780.9; oleandrin, 576.7; and lanatoside B,

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⁴ Kindly supplied by E. Merck, Darmstadt, Germany.

⁵ Kindly supplied by Wellcome Research Laboratories, Tuckahoe, New York.

⁶ Kindly supplied by Schering Corporation, Bloomfield, New Jersey and marketed as Olnerin, also known as Folinerin in some foreign countries.

⁷ Kindly supplied by Sandoz Chemical Works, Inc., New York, N. Y.

1074. Stock solutions containing 1 mgm. of the glycoside in 1 cc. of 95 per cent ethyl alcohol were diluted to the desired concentration in 0.9 per cent sodium chloride solution. All solutions were infused into the venous supply cannula of the HLP by means of a constant infusion pump.⁸

The HLP was prepared by the method of Patterson and Starling (8) and the set-up was essentially the same as that used by Krayner and Mendez (9). Mongrel dogs weighing between 7.0 and 14.8 kg. were employed and in all, 56 heart-lung preparations were used in this study. In all the experiments with digoxin, oleandrin, and lanatoside B, right and left atrial pressure, pulmonary and aortic arterial pressure, systemic output and heart rates were recorded. Right and left atrial pressures were recorded with water manometers whereas pulmonary arterial pressure was recorded with a bromoform manometer. In the experiments with digitoxin and g-strophanthin pulmonary and left atrial pressures were not recorded. Aortic pressure was recorded from a side branch of the arterial cannula by means of a mercury manometer. The systemic output was recorded with a Weese Stromuhr (10). Heart rates were counted directly every few minutes. Blood temperature was between 38.5 and 39.3°C. The artificial resistance was at 75 mm. of mercury. The blood volume was 850-900 cc. at the beginning of the experiment.

Wood and Moe (11) have shown that in the HLP the heart increases in weight due to edema formation. To determine the true cardiac weight, the method of chloride space determinations as recommended by Wood and Moe (11) has been used. In all the experiments with digoxin, oleandrin, and lanatoside B this correction has been applied. The experiments with g-strophanthin and digitoxin were obtained earlier and no chloride space determinations were made. However, for purposes of comparison, the "true heart weight" was estimated by multiplying the body weight in kg. by the factor 0.84 (7). For all calculations the corrected heart weights were used.

Marked therapeutic action of cardiac glycosides can only be seen in the failing heart. In the heart-lung preparation the most commonly employed types of heart failure are spontaneous failure and heart failure produced by a barbiturate or some other toxic agent. In our experiments we usually allowed the development of a slight spontaneous failure by waiting 30-60 minutes following the completion of the HLP. While at that time cardiac failure was slight, we were able in every experiment to demonstrate definite cardiac improvement following the infusion of a cardiac glycoside. In a number of experiments heart failure was produced with sodium pentobarbital given into the venous supply reservoir in doses of 100-180 mg.

RESULTS. In the heart-lung preparation of the dog the left and right atrial pressures do not fall spontaneously, but gradually rise throughout the course of the experiment. Accompanying these rises of pressure there is within two to three hours a gradual fall in systemic output and a 10 to 30 per cent decrease in heart rate. The infusion of a cardiac glycoside results in a fall of the right and left atrial pressures, an increase in the systemic output, and a more pronounced decrease in the heart rate. Following the administration of a cardiac glycoside the left atrial pressure consistently falls earlier and to a greater extent than the right atrial pressure. A fall of atrial pressure with constant venous supply and without a change in heart rate is a sensitive indicator of improvement in cardiac contractility (12). As a more rigorous criterium of improvement in cardiac function, competence tests were made as suggested by Krayner (12). The reduction in atrial pressures, the increased systemic output, and the competence tests were all used to assess the therapeutic effect produced by the

⁸ Constructed by K. Kniazuk, Merck Instituto for Therapeutic Research, Rahway, New Jersey.

infusion of the cardiac glycosides. Following the appearance of therapeutic effects the continued infusion of a cardiac glycoside results in cardiac irregulari-

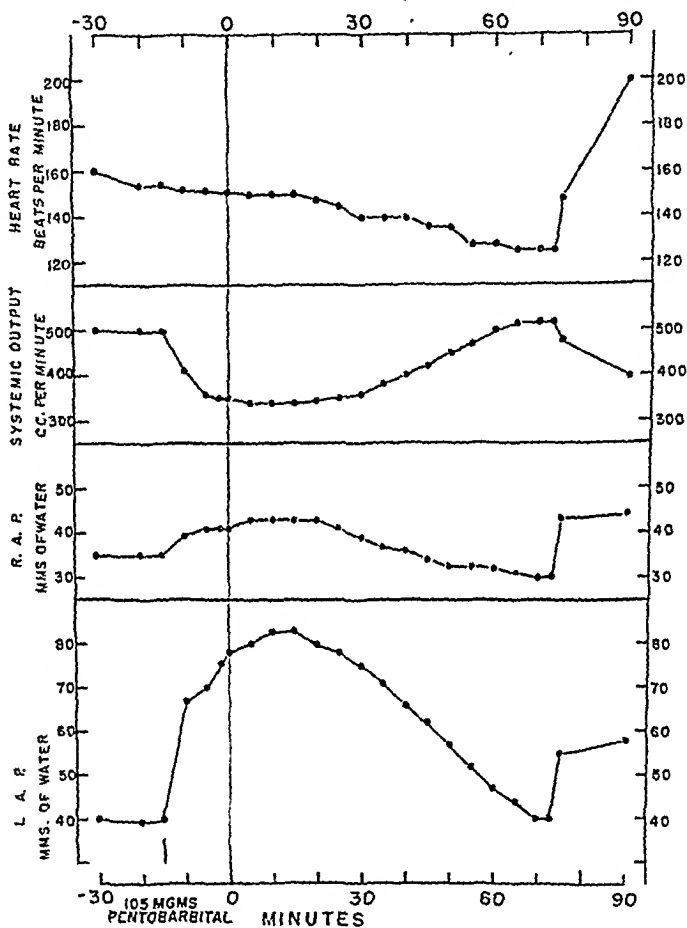


FIG. 1. THE EFFECT OF A CONSTANT INFUSION OF DIGOXIN ON PENTOBARBITAL HEART FAILURE IN THE H.L.P. OF THE DOG

HLP. Male dog, 9.5 kg. Anesthesia 35 mg. pentobarbital sodium per kg., given intraperitoneally; peripheral resistance 75 mm. Hg.; blood temp. 38.8-39.2° C. Blood volume 900 cc. Heart weight 69.5 grams. At minus 15 minutes 105 mg. of pentobarbital sodium was given into the venous reservoir. At 0 time a constant infusion of digoxin was started at a rate of 0.13 micromols per kg. heart per minute. R.A.P. Right atrial pressure. L.A.P. Left atrial pressure.

ties. These irregularities are accompanied by a sharp increase in heart rate, a concomitant rise of atrial pressures, and a reduction in the systemic output (fig. 1 and 2). Further continuation of the infusion eventually results in ventricular fibrillation.

The therapeutic dose was considered to be that amount of a cardiac glycoside which just produced a reduction in atrial pressure (12). Using the decrease of

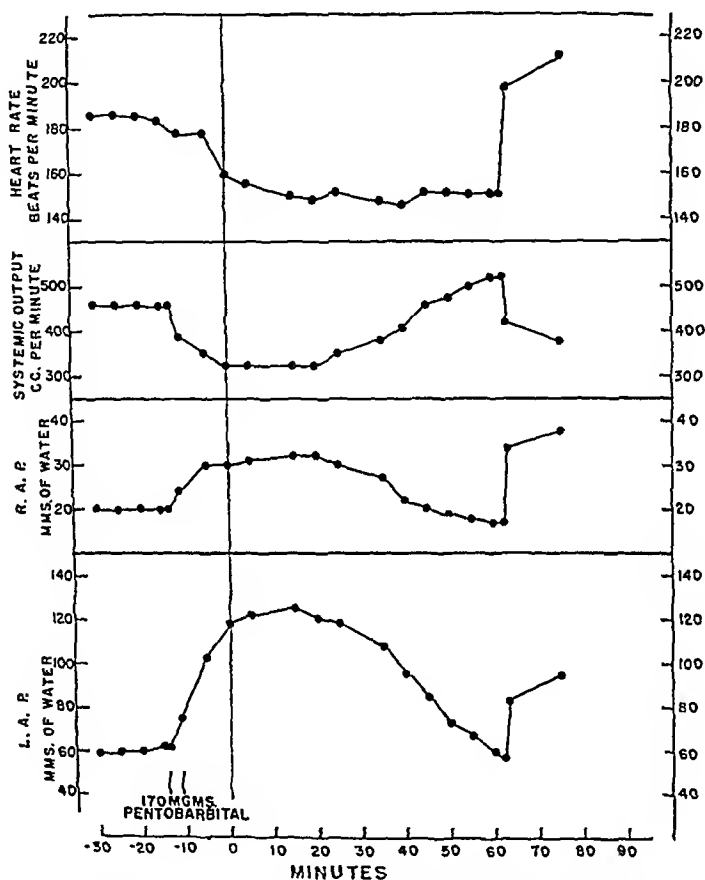


FIG. 2. THE EFFECT OF A CONSTANT INFUSION OF LANATOSIDE B ON PENTOBARBITAL HEART FAILURE IN THE H.L.P. OF THE DOG

HLP. Male dog, 9.2 kg. Anesthesia 35 mg. per kg. pentobarbital sodium given intraperitoneally; peripheral resistance 75 mm. Hg. Blood temp. 38.8-39.2° C. Blood volume 900 cc.; Heart weight 55.5 grams. At minus 13 minutes 170 mg. pentobarbital sodium was given into the venous reservoir. At 0 time a constant infusion of lanatoside B was started at a rate of 0.71 micromols per kg. heart per minute. R.A.P. Right atrial pressure. L.A.P. Left atrial pressure.

right atrial pressure as an indicator usually yielded a higher therapeutic dose than using the reduction of left atrial pressure, because decrease of left atrial pressure is a more sensitive indicator of cardiac improvement. The amount of glycoside infused up to the moment irregularities first appeared was considered

the irregularity dose, while the amount producing ventricular fibrillation was designated the lethal dose. The time from the beginning of the infusion to the appearance of ventricular fibrillation we refer to as the experimental time.

Figures 1 and 2 show the characteristic results. In both experiments heart failure was produced by an injection of pentobarbital sodium. The infusion of digoxin (fig. 1) at a rate somewhat higher than the optimal (1) resulted in a reduction of the atrial pressures within 15 minutes after the start of the infusion. The improvement in cardiac function is also seen in the increase of the systemic output. The hemodynamic characteristics of the heart were similar to those before heart failure was produced. It can be seen from a comparison of figure 2

TABLE 1
*g-Strophanthin**

RATE OF ADMINISTRATION	THERAPEUTIC DOSE RIGHT ATRIAL PRESSURE		IRREGULARITY DOSE		LETHAL DOSE	EXPERIMENTAL TIME
	micromols per Kg. heart per minute	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	in minutes
0.762	1.00	18	7.00	67	10.48	14
0.579	2.27	16	9.05	65	13.87	24
0.375	2.15	21	5.84	55	10.51	28
0.202	1.69	20	5.08	60	8.46	42
0.111	0.73	12	4.29	70	6.11	55
0.048	0.67	21	1.91	60	3.20	67
0.033	0.71	22	2.15	67	3.23	98
0.017	0.40	17	1.45	61	2.38	139
0.016	0.31	18	1.04	60	1.73	107
0.012	0.54	21	1.44	56	2.57	213
0.010	0.49	25	1.24	64	1.94	195
0.007	0.33	17	1.30	66	1.97	280

* Part of these data have been used in a previous publication (7).

with figure 1 that no qualitative differences between lanatoside B and digoxin can be detected.

In tables 1 to 5 are given for the five glycosides studied, rate of administration, therapeutic dose, irregularity dose, lethal dose, and experimental time. As with digitoxin and *g-strophanthin*, the rate of administration has a marked effect on the lethal dose of the glycosides digoxin, oleandrin, and lanatoside B. Furthermore, the rate of administration influences the therapeutic dose and irregularity dose in the same manner as the lethal dose. In figure 3, for example, it can be seen that the reduction in the rate of administration of oleandrin from 1.6 to 0.08 micromols per kg. per minute decreases the therapeutic dose and the irregularity dose as well as the lethal dose. A further reduction of the rate of administration does not alter these values significantly. The *optimal rate of administration* has been previously defined (7) as the highest rate of administra-

tion with which the minimal lethal dose can still be determined. In analogy with the definition of *minimal lethal dose* the values of the therapeutic and irregu-

TABLE 2

*Digitoxin**

RATE OF ADMINISTRATION	THERAPEUTIC DOSE RIGHT ATRIAL PRESSURE		IRREGULARITY DOSE		LETHAL DOSE	EXPERIMENTAL TIME
	micromols per Kg. heart per minute	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	in minutes
0.942	11.68	20	35.05	60	58.43	62
0.706	9.65	18	32.84	61	53.67	76
0.577	9.55	19	28.87	58	49.77	86
0.415	7.35	16	21.42	61	35.13	83
0.384	6.22	20	20.73	67	31.17	81
0.323	3.77	15	17.01	68	24.98	78
0.217	—	—	15.21	65	23.39	108
0.172	3.79	20	11.11	59	18.79	109
0.104	2.46	18	8.59	63	13.55	131
0.068	1.83	16	7.14	62	11.58	170
0.044	2.06	23	4.69	58	8.09	183
0.034	1.21	15	4.70	57	8.20	237
0.031	1.38	20	4.63	65	7.09	230
0.024	1.49	21	4.13	58	7.09	286
0.019	1.43	19	4.58	61	7.51	375

* Part of these data have been used in a previous publication (7).

TABLE 3

Oleandrin

RATE OF ADMINISTRATION	THERAPEUTIC DOSE				IRREGULARITY DOSE		LETHAL DOSE	EXPERIMENTAL TIME
	Left Atrial Pressure		Right Atrial Pressure		micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	in minutes
	micromols per Kg. heart per minute	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose				
1.614	8.063	16	9.01	18	28.25	57	49.24	30
0.560	3.624	13	4.70	17	17.63	63	27.98	50
0.480	2.878	13	5.84	27	15.13	70	21.62	45
0.228	2.288	14	3.62	21	10.33	61	17.01	74
0.215	2.150	13	3.42	20	10.24	60	17.04	79
0.102	1.821	16	2.24	20	8.26	73	11.30	111
0.076	0.919	10	1.21	13	5.05	53	9.48	124
0.049	0.978	22	1.37	15	4.78	57	9.05	185
0.036	1.023	11	1.68	17	5.20	53	9.71	266

larity dose obtained with rates of administration at or below the optimal rate of administration have been designated the *minimal therapeutic dose* and *min-*

TABLE 4

Digoxin

RATE OF ADMINISTRATION	THERAPEUTIC DOSE				IRREGULARITY DOSE		LETHAL DOSE	EXPERIMENTAL TIME	REMARKS
	LEFT ATRIAL PRESSURE		RIGHT ATRIAL PRESSURE		micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	in minutes	
	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose					
micromols per Kg. heart per minute	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart		
0.485	2.43	11	3.39	16	15.80	72	21.89	45	Pentobarbital heart failure
0.226	2.15	15	2.47	18	9.08	65	13.97	61	
0.133	1.80	18	2.01	20	6.34	64	9.89	74	
0.088	0.97	10	1.95	20	6.01	62	9.74	119	Pentobarbital heart failure Severe spontaneous failure
0.069	1.04	—	1.04	—	5.17	—	—	—	
0.061	0.96	13	1.25	18	3.82	54	7.05	118	
0.058	0.46	7	0.63	10	4.27	67	6.34	110	
0.052	1.04	16	1.20	18	4.20	64	6.52	125	
0.050	0.60	—	0.60	—	3.49	—	—	—	
0.033	0.46	11	0.55	13	3.08	72	4.30	131	
0.025	0.37	8	0.61	12	2.54	52	4.93	198	
0.016	0.36	10	0.43	11	2.02	53	3.81	245	

TABLE 5

Lanatoside B

RATE OF ADMINISTRATION	THERAPEUTIC DOSE				IRREGULARITY DOSE		LETHAL DOSE	EXPERIMENTAL TIME	REMARKS
	Left Atrial Pressure		Right Atrial Pressure		micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	in minutes	
	micromols per Kg. heart	per cent of lethal dose	micromols per Kg. heart	per cent of lethal dose					
1.17	14.20	15	14.66	16	59.6	63	94.00	82	Pentobarbital heart failure
0.714	9.36	12	13.4	17	42.11	53	79.30	111	
0.59	7.65	15	10.61	21	32.93	66	50.05	85	
0.48	5.72	13	5.72	13	27.24	63	43.04	90	
0.304	6.42	11	9.14	16	38.45	63	56.80	186	
0.288	4.18	17	8.35	14	37.16	62	59.65	207	
0.175	6.61	19	7.66	22	19.33	56	34.83	198	
0.11	4.11	12	5.96	18	21.03	63	33.65	301	

imal irregularity dose. Table 6 shows the values obtained for the five glycosides studied.

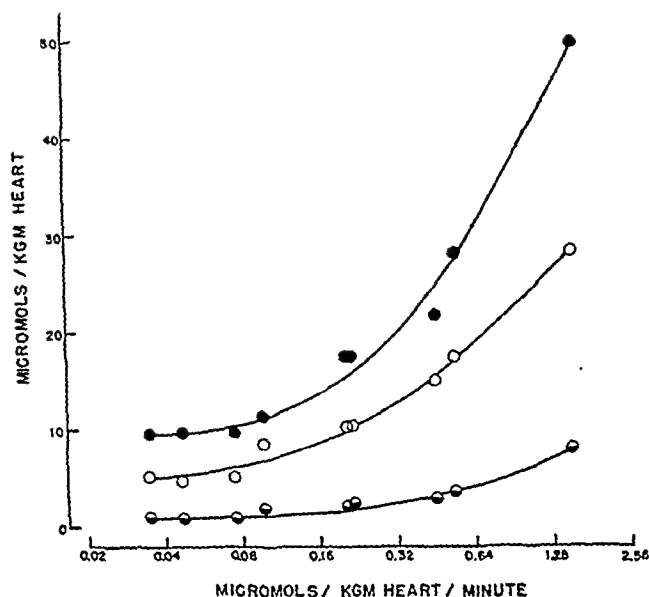


FIG. 3. THE INFLUENCE OF RATE OF ADMINISTRATION ON THE THERAPEUTIC DOSE, IRREGULARITY DOSE, AND LETHAL DOSE OF OLEANDRIN IN THE H.L.P. OF THE DOG

- = therapeutic dose
○ = irregularity dose
● = lethal dose

Abscissa: Rate of administration in micromols per kg. heart per minute.

Ordinate: Effective dose in micromols per kg. heart.

TABLE 6

	OPTIMAL RATE OF ADMINISTRATION	OPTIMAL EXPERIMENTAL TIME	MINIMAL THERAPEUTIC DOSE		MINIMAL IRREGULARITY DOSE	MINIMAL LETHAL DOSE
	micromols per Kg. heart per minute	minutes	Left Atrial Pressure micromols per Kg. heart	Right Atrial Pressure micromols per Kg. heart	micromols per Kg. heart	micromols per Kg. heart
G Strophanthin.	0.018-0.015	120-140	—	0.35	1.29	2.12
Digoxin.	0.036-0.031	120-140	0.43	0.65	2.54	4.34
Digitoxin .	0.034-0.031	220-740	—	1.20	5.01	7.47
Oleandrin .	0.078-0.063	120-150	0.97	1.50	5.01	9.41
Lanatoside B	about 0.17	about 200	4.4	6.00	20.18	34.20

The influence of the rate of administration on the ratio of irregularity dose to therapeutic dose and on the ratio of lethal dose to irregularity dose has been somewhat variable. The data given in tables 1 to 5 show no consistent relation between the rate of administration and either of these ratios.

In figure 4 average values of the therapeutic and irregularity dose have been expressed in per cent of the lethal dose. It can be seen from this figure that all the five glycosides studied have about the same ratio of lethal dose to irregularity dose (LD/ID) and lethal dose to therapeutic dose (LD/TD).

As determined under our experimental conditions the relative therapeutic dose is 12 to 14 per cent of the lethal dose when the left atrial pressure is used, and 16 to 19 per cent of the lethal dose when the right atrial pressure is used as criterium⁷ of therapeutic effect. The relative irregularity dose varies between

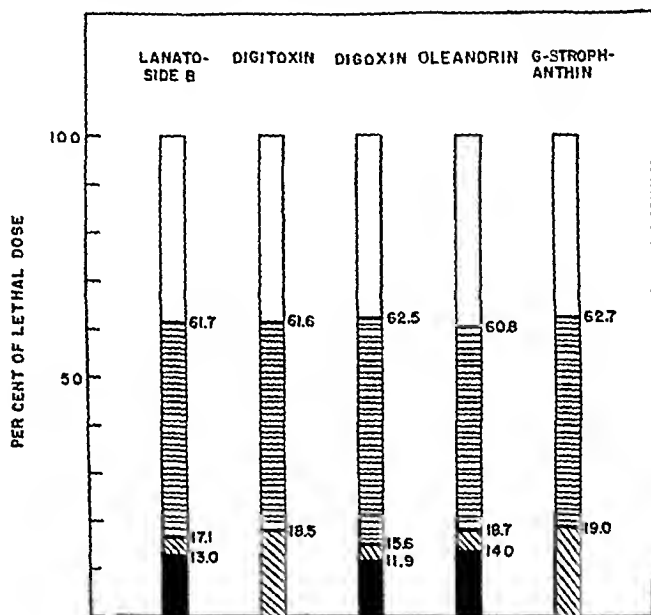


FIG. 4. THE RELATIVE ACTIVITY OF VARIOUS CARDIAC GLYCOSIDES IN THE H.L.P. OF THE DOG

Solid black: Average therapeutic dose as determined on left atrial pressure.

Diagonal cross hatching: Average therapeutic dose as determined on the right atrial pressure.

Horizontal cross hatching: Average irregularity dose.

61 and 63 per cent of the lethal dose. Lanatoside B (see figure 4) does not differ from the four other glycosides studied.

DISCUSSION. It has been shown previously (7) and in the present investigation that for a number of cardiac glycosides the rate of administration is an important factor in determining the therapeutic, toxic, and lethal dose. All these values tend to become constant when rates less than the optimal are being employed. A comparison of the values of the MLD show that on a molar basis potency as regards therapeutic and toxic action decrease in the following order:

g-strophanthin, digoxin, digitoxin, oleandrin, lanatoside B. This order of activity is different from that determined for the intact cat (13). Similar discrepancies between intact animal and isolated organs have been reported by Cattell and Gold (14). These discrepancies between the isolated dog heart and the intact cat may be due to species differences of cardiac tissue or to a difference in the extra-cardial distribution of these glycosides or to the differences in innervation (15). A final answer to this question must await the determination of the activity of these glycosides in the intact dog and in the cat HLP.

In the present series of experiments the therapeutic dose was about 15 per cent of the lethal dose and about 25 per cent of the irregularity dose for all the glycosides studied (see figure 4). Of special interest are the results with lanatoside B which, contrary to the findings of Moe and Visscher (2), has a relative therapeutic dose equal to 13 per cent of the lethal dose. The discrepancy may be due to differences in technique, since Moe and Visscher utilized efficiency determinations at constant diastolic volume as a criterium of therapeutic effects. Furthermore, they gave relatively large successive single doses within a short time. It is possible that with the single dose technique the therapeutic effects of the slower-acting glycosides, such as lanatoside A and B, are obscured by the progressing heart failure. The differences between various glycosides observed by Moe and Visscher may be a reflection of the differences in speed of action of these glycosides rather than differences in therapeutic range.

Takahashi et al. (4) have used ventricular suspension curves in the intact rabbit as an index of therapeutic action. This is not a satisfactory method for determining cardiac improvement since a number of other factors influence such a recording. Furthermore, in the rabbit the elimination of g-strophanthin is much faster than that of digitoxin (16) and may have influenced their results.

SUMMARY

The influence of rate of administration on the therapeutic, irregularity, and lethal dose of the glycosides digitoxin, g-strophanthin, digoxin, oleandrin, and lanatoside B has been studied in the HLP of the dog. A reduction of the rate of administration results in a reduction of all these values until the optimal rate of administration is reached. At and below the optimal rate of administration, the minimal therapeutic dose, minimal irregularity dose, and minimal lethal dose are determined. On a molar basis the cardiac glycosides studied have the following order of decreasing potency: g-strophanthin, digoxin, digitoxin, oleandrin, and lanatoside B.

The average ratios of irregularity dose to therapeutic dose and lethal dose to therapeutic dose are the same for all the five glycosides studied. The therapeutic dose is about 15 per cent and the irregularity dose is approximately 60 per cent of the lethal dose.

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THE ABSORPTION, DISTRIBUTION AND EXCRETION OF STREPTOMYCIN¹

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Numerous studies on the absorption, distribution and excretion of streptomycin in the dog (1, 2) and in man (3, 4, 5, 6, 7) have been reported. The data obtained in these studies pertinent to the present investigation may be summarized as follows. After intravenous injection maximum serum concentrations are obtained in a few minutes, whereas after intramuscular injection of the same dose there is a rise to a maximum in one to two hours. When the maximum is reached for intramuscular injection, the serum concentrations are approximately the same as the levels obtained at the same time interval for intravenous administration and there is then a gradual decline of the serum concentrations. In the dog, 23-80 per cent of the streptomycin is excreted in the urine; in man, urinary excretion is reported to vary from 6 to 90 per cent with most values around 50-60 per cent. The renal plasma clearance for man varied from 38-67 cc. per minute. In addition, the data reported would indicate that streptomycin is distributed in extracellular water only.

The method used for the determination of streptomycin in all the above studies has been a biological one depending on the antibacterial properties of streptomycin. The development of chemical methods for the determination of streptomycin in plasma and in urine allowed a study to be made with these methods. The results obtained are reported in the present communication.

After this study was nearly complete, Boxer, Jelinek and Leghorn (8) using a chemical method reported the excretion of 88 and 89 per cent of injected streptomycin in two dogs in the course of 6 to 8 hours.

METHODS. A single lot of streptomycin was used for the entire study. This was a sample of purified streptomycin sulfate. It had a potency of 824 meg./mg. Chemical analysis gave 77 per cent of base and counter current distribution studies indicated the sample to be mainly streptomycin A and to contain less than 5 per cent streptomycin B (9)².

The methods used for determination of streptomycin in plasma and urine were those described by Marshall, Blanchard and Buhle (9). All dosages and figures for plasma and urine are reported as streptomycin base.

Four female dogs and five male patients were used in the study. The patients were free of hepatic, renal and cardiac disease. In the dogs, streptomycin was given in 10 cc. of water intravenously in the course of 3-4 minutes; in man, intravenous streptomycin was given in

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² We wish to thank Dr. R. A. Patelski of Chas. Pfizer and Co. for a generous supply of streptomycin sulfate, and Dr. O. Wintersteiner of the Squibb Institute for Medical Research for the data on the Craig counter-current distribution study.

100 cc. of saline over 10 minutes. Urine was collected from the dogs by catheter; in man, by voiding. Blood was drawn from a vein, oxalated, and centrifuged at once to obtain plasma.

TABLE 1
Apparent volume of distribution of streptomycin in per cent of body weight

DOG	EXPERIMENT	CALCULATED FROM	
		Zero time	One hour
1	1	23	25
	2	31	
	3	29	31
2	1	34	36
PATIENT			
A	1	30	32
J	1	31	35
E	1	31	31
	2	35	

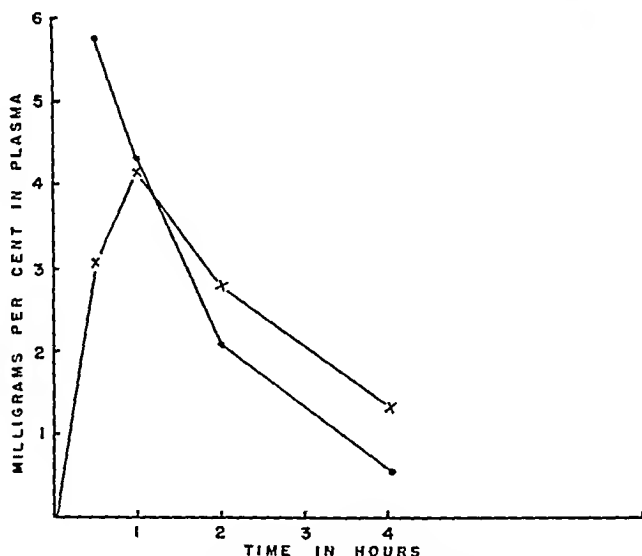


FIG. 1. INJECTION IN DOG 1 OF 20 MILLIGRAMS PER KILOGRAM OF STREPTOMYCIN
●—● intravenously; X—X intramuscularly

RESULTS. *Distribution.* Streptomycin is distributed as if it were present in extracellular water only. After intravenous injection of 20 mg. per kilogram in dogs and 10-20 mg. per kilogram in patients, plasma concentrations were deter-

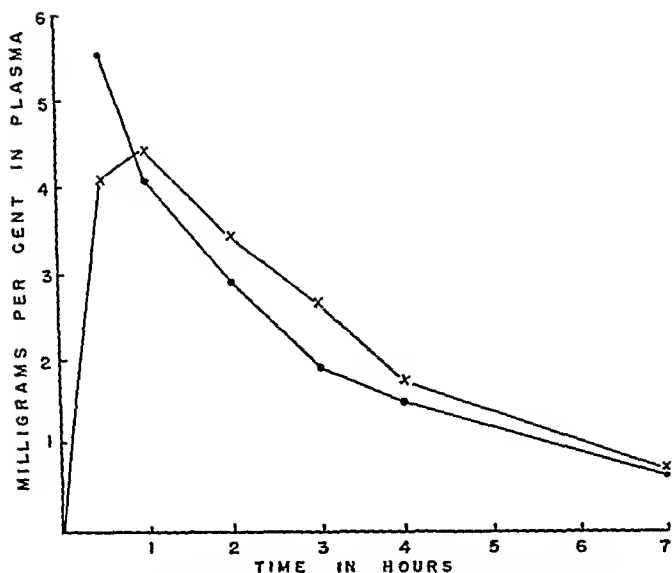


FIG. 2. INJECTION IN PATIENT A OF 20 MILLIGRAMS PER KILOGRAM OF STREPTOMYCIN

●—● intravenously; X—X intramuscularly

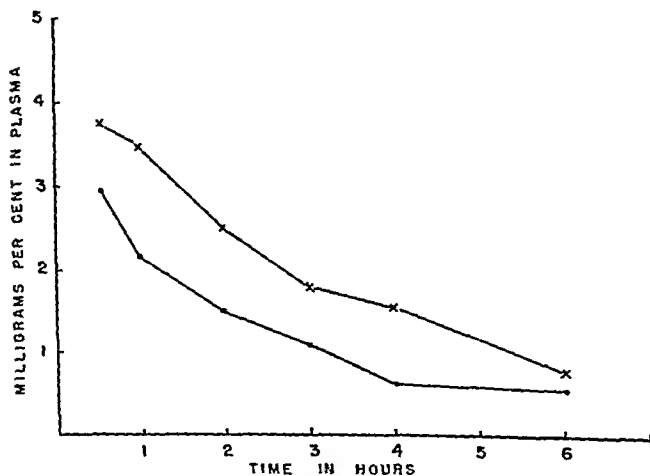


FIG. 3. INJECTION OF STREPTOMYCIN INTRAVENOUSLY INTO PATIENT E

●—● 10 milligrams per kilogram; X—X 20 milligrams per kilogram

mined at one-half, one, two, three and four hours. The apparent volume of distribution in per cent of body weight was calculated from the data obtained in

two ways. The logarithms of plasma concentrations were plotted against time, and the straight line obtained extrapolated to zero time. The value obtained together with the dose given per kilogram allowed a calculation of the distribution in per cent of body weight. A slight error is introduced by assuming that the rate of elimination of the substance from the body is the same in the first one-half hour as in succeeding hours. The other method used was to calculate the distribution from the one-hour plasma concentration assuming that the amount of drug excreted in the urine during the first hour represents the total amount disappearing from the body in that time. The results of these calculations are given in table 1.

TABLE 2
Excretion of streptomycin in urine

DOG	DOSE	ROUTE	EXCRETED IN % AMOUNT INJECTED IN	
			6 or 8 hrs.	24 hrs.
1	mg./kg.			
	20	Intravenous	80	88
	20	Intramuscular	79	
20	Intravenous	76		
2	20	Intravenous	70	86
3	30	Intravenous	74	
4	20	Intravenous	68	
PATIENT				
A	20	Intravenous	50	59
	20	Intramuscular	55	59
J	14	Intravenous	56	83
E	20	Intravenous	68	
H	15	Intramuscular	56	

Plasma-concentration time curves. The results of experiments to determine the plasma-concentration time curves of streptomycin after intravenous and intramuscular injection in the dog and in man are illustrated by data plotted in figures 1, 2 and 3. Since at one hour after intramuscular injection the plasma concentration closely approximates that obtained from intravenous injection of the same dose, it appears that absorption from the muscles is rapid.

Excretion. Data on the total excretion of streptomycin in the urine after intravenous and intramuscular injection in the dog and in man are summarized in table 2. In the case of the dog the excretion is given for 6 and for 24 hours; in the case of man, for 8 and 24 hours. The total amount excreted appears to be somewhat less in man than in the dog.

The renal clearance of streptomycin has been calculated from the data obtained for both the dog and man. Plasma concentrations ranged from 0.7 to 5.0 milligrams per 100 cc. A summary of the values is given in table 3.

DISCUSSION. The data obtained in this investigation of the absorption,

distribution and excretion of streptomycin in the dog and in man are in general in agreement with those obtained previously using the biological method of assay for streptomycin. The results on the excretion of streptomycin in the urine are more consistent than those obtained previously. The importance of checking results obtained with the biological method by those obtained with a chemical method is, however, obvious.

In the dog, nearly all of the streptomycin administered can be accounted for by excretion in the urine. In man, a considerable amount of the streptomycin is frequently not found in the urine. The renal clearance of streptomycin in both dog and man is less than the reported values for the glomerular clearance. Unless streptomycin is bound to a great extent by the proteins of plasma, it would appear to be excreted by glomerular filtration alone.

TABLE 3
Renal clearance of streptomycin

DOG	EXPERIMENT	WEIGHT	CLEARANCE, CC. PLASMA PER MINUTE
		kg.	
1	1	24	45, 44, 53
	2		56, 47
	3		59, 53, 55
2	1	24	38, 35, 35, 34
PATIENT			
A	1	50	49, 56, 45
	2		47
J	1	73	60, 80, 71
E	1	50	35, 30, 42

The assumption has been made in this work that the substance determined in plasma and urine is the same chemical compound which is administered. No conclusive proof of this is available at present. However, neither streptobiosamine nor dihydrostreptomycin are determined by the methods used.

SUMMARY

Streptomycin has been determined by a chemical method in plasma and urine after intravenous and intramuscular injection in the dog and in man. It appears to be distributed in a percentage of the body weight which approximates extracellular water. Nearly all of the drug injected can be accounted for by excretion in the urine in the dog; this is not always true in the case of the human subject. The renal plasma clearance in both dog and man is lower than the glomerular clearance, where no allowance is made for a possible plasma binding of streptomycin.

We wish to thank Mildred Salchunis for technical assistance, and Dr. C. G. Zubrod for aid in the patient studies.

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THE METABOLIC FATE OF CHLORAL HYDRATE

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In 1882 von Mering (1) showed that urochloralic acid, the substance that he and Musculus seven years earlier (2) had isolated from the urine of patients receiving chloral hydrate, was a conjugation product of trichloroethanol with glucuronic acid. In 1884 Külz (3) demonstrated that the alcohol was itself narcotic and that it too gave rise to the same conjugation product, urochloralic acid. Akamatsu and Wasmuth (4) in 1923, having measured the conjugated glucuronic acid in the urine of rabbits, concluded that chloral hydrate and trichloroethanol are both converted to the conjugated product in about the same proportions (about half). These results strongly suggest that chloral hydrate is initially reduced in large part to trichloroethanol, some of the latter compound then being excreted in the conjugated form.

2,2,2-Trichloroethanol is a substance which, on intravenous injection, appears to be a more active narcotic than chloral hydrate. It is a substance the physical properties of which are more consistent with those of other typical narcotics of comparable activity than are those of chloral hydrate. The question then arises whether the trichloroethanol produced by the reduction of chloral hydrate may not play some part in the depressant phenomena that follow the administration of chloral hydrate, or whether it may not even be entirely responsible for them. Although the facts suggesting this question have been known for many years, the role of trichloroethanol in chloral hydrate narcosis has not hitherto been clarified. Even if chloral hydrate were assumed to be completely reduced to the alcohol, it could not be assumed that any significant amount of free trichloroethanol would be present at any one time unless there were some knowledge of the relative rates of the reactions responsible for its production and for its inactivation. This information has been lacking. That chloral hydrate is even reduced prior to conjugation has been questioned (5).

In the present investigation an attempt has been made to throw some light on these questions by measuring the free trichloroethanol in plasma following the administration of chloral hydrate and of equivalent amounts of the alcohol itself. The development of a chemical method for the determination of trichloroethanol in the presence of chloral hydrate, urochloralic acid, and trichloroacetic acid has made this possible. There have apparently been no previous attempts to measure trichloroethanol in blood or tissues. Determinations of chloral hydrate and trichloroacetic acid have also been included in the present work. Most of the analyses that have been reported for chloral hydrate (6-10) were made by unspecific methods that probably included at least a part of the trichloroethanol and urochloralic acid present as well as the chloral hydrate.

The method of Friedman and Calderone (11), which was used by Mukerji and Ghose (12) to determine chloral hydrate in blood, utilizes the same reaction as the method employed here, but would determine trichloroacetic acid as well as chloral hydrate. So far as I am aware, trichloroacetic acid has not heretofore been searched for as a metabolic product of chloral hydrate.

The methods employed in this work for the determination of trichloroethanol, chloral hydrate, and trichloroacetic acid are all based upon a reaction discovered by Fujiwara (13) and later independently by Ross (14). Chloroform, chloral hydrate, trichloroacetic acid, and a number of other trihalogen compounds, when heated with pyridine and an aqueous solution of sodium hydroxide, give rise to a crimson color in the pyridine layer. The reaction is quite sensitive, quantities of these substances of the order of 1 microgram giving a visible color. It is suitable for quantitative work and has been used for the determination of chloroform (15-20), trichloroethylene (18, 19, 21-24), chloral hydrate (11, 12), carbon tetrachloride (17, 19), and trichloroacetic acid (23, 25).

Trichloroethanol does not itself give the Fujiwara reaction. The method used here for its determination depends upon its removal from plasma and separation from the interfering substances known or presumed to be present by means of a multiple extraction process, its oxidation to trichloroacetic acid by dichromate, and the subsequent application of the Fujiwara reaction. Chloral hydrate and trichloroacetic acid are both determined by a direct application of the Fujiwara reaction to deproteinized plasma. The two substances are differentiated by the fact that chloral hydrate is rapidly and completely decomposed in strongly alkaline solution at room temperature, the chloroform produced in the reaction being removable by extraction. Under the same conditions, trichloroacetic acid is not significantly altered. This allows the independent determination of chloral hydrate and trichloroacetic acid in the presence of each other, and in this respect the present methods differ from the previous applications of the Fujiwara reaction to the determination of chloral hydrate and trichloroacetic acid.

MATERIALS. The trichloroethanol used was synthesized six years previously in the laboratories of the Mallinckrodt Chemical Works. It was dried with sodium sulfate and redistilled (b. p. 80°/50 mm.) just prior to the present use and thereafter protected from light and moisture. Chloral hydrate was purchased from Eastman Kodak Co. Titration by the method of the U. S. P. XIII showed no evidence of impurity. The trichloroacetic acid was Mallinckrodt "Analytical Reagent". The concentrations of solutions of trichloroacetic acid used for standardization of the method and for injection into the dogs were determined by titration. "Heptane" was Eastman "practical" (from petroleum) treated with sulfuric acid and permanganate and redistilled. This purification was necessary in order to prevent oxidizable materials from being carried over into the final extract in sufficient quantities to reduce all of the dichromate. "Pentane" was Eastman "practical" (from petroleum).

CHEMICAL METHODS. The methods described below permit analyses for trichloroethanol, chloral hydrate, and trichloroacetic acid to be performed on a single sample of 2 ml. of plasma and are suitable for determining concentrations of the three substances as low as about 10 mgm. per l. This degree of sensitivity was adequate for the present investigation. The methods can, however, be modified to give greater sensitivity.

The color produced in the Fujiwara reaction both by chloral hydrate and by trichloroacetic acid is crimson, the absorption band extending over the mid-portion of the visible spectrum with the maximum at about 525 m μ . The color fades rather rapidly, and its intensity should be measured within a few minutes of its development. In the work reported here a photoelectric photometer with a Wratten 54 filter was used for this measurement.

Coagulation of the blood samples was prevented with potassium oxalate. The plasma was separated within half an hour and the initial extraction with heptane was carried out within five hours of the time the blood was drawn.

Determination of 2,2,2-Trichloroethanol in Plasma. A sample of 2 ml. of plasma is mixed with an equal volume of water and shaken with 35 ml. of "heptane". After centrifugation, 30 ml. of the heptane layer is removed and shaken with 16 ml. of water. After centrifugation, 15 ml. of the water layer is removed. In this is dissolved 5 gm. of sodium chloride, and the solution is shaken with 5 ml. of "pentane". Of the pentane layer, 4 ml. is transferred to a test tube, and 0.1 ml. of pyridine added. The tube is immersed in an ice bath and the pentane removed by drawing a stream of dry air over the surface of the liquid through a fine capillary, the pressure in the tube being reduced to about 200 mm. Hg. The disappearance of practically all of the pentane is marked by the cessation of creeping of liquid up the walls of the tube. The residue then consists largely of pyridine, and contains almost all of the trichloroethanol that was in the pentane solution.¹ To this residue is added 0.5 ml. of a solution consisting of: potassium dichromate, 1 gm.; water, 45 ml.; concentrated sulfuric acid, 55 ml. The tube is now kept at 20° C. for 1 hr., after which time it is immersed in an ice bath and 4 ml. of 5.5 N sodium hydroxide is added slowly with stirring.² After the addition of 5 ml. of pyridine, the tube is immersed in a bath of boiling water for 2 min., during which time the contents are stirred continuously. The tube is immediately cooled in an ice bath. From the upper phase, 4 ml. is transferred to a cuvette and diluted with 1 ml. of water. The optical density of this solution is a linear function of the concentration of trichloroethanol in the plasma.³ Determinations are reproducible within limits not exceeding a 10 per cent deviation from the mean.

Determination of Chloral Hydrate and Trichloroacetic Acid in Plasma. These substances can be determined in the plasma extracted for the trichloroethanol analysis, since they are not removed in significant amounts by heptane. To 2 ml. of the diluted and extracted plasma from the former analysis is added 8 ml. of the tungstic acid solution of Van Slyke and Hawkins (26). After centrifugation, 1 ml. of the supernatant liquid is transferred to each of two tubes. To the first tube 4 ml. of 8 N sodium hydroxide is added and the resulting mixture shaken twice with 5 ml. portions of "heptane", which are discarded. Then 5 ml. of pyridine is added and the tube heated for 2 min. as in the trichloroethanol analysis. Subsequent procedures are the same as in that analysis. The color is all due to trichloroacetic acid, the chloroform produced by the decomposition of chloral hydrate having been removed by the heptane.

To the second tube 5 ml. of pyridine is added and then 4 ml. of 8 N sodium hydroxide. The tube is then heated as in the other procedures. Chloral hydrate and trichloroacetic acid both contribute to the color in this tube, and chloral hydrate is determined from the difference between this and the first tube.

¹ Unless pyridine is added, it is difficult to evaporate very dilute solutions of trichloroethanol without the loss of a large proportion of the trichloroethanol with the solvent vapor.

² The yield of trichloroacetic acid under these conditions is only about 80 per cent of the theoretical, but is sufficiently reproducible for the present purposes.

³ In the extraction process no attempt is made to conserve all the trichloroethanol in the plasma. Although only a small proportion of that originally in the plasma sample appears in the final extract, the amount there will be proportional to the plasma concentration if the distribution coefficients are independent of concentration. Within the limits of error of the method, this has been found to be the case.

THE CHEMICAL TRANSFORMATIONS OF CHLORAL HYDRATE IN THE DOG. Two dogs were given chloral hydrate intravenously. In both experiments (figs. 1 and 4), the concentration of chloral hydrate itself in the plasma declines at a rapid rate and soon reaches a level undeterminable by the present method. Trichloroethanol and trichloroacetic acid are both present in considerable concentrations in the first samples taken after the injections. They rise for the next few minutes. The trichloroethanol concentration then falls rather slowly at a rate proportional to the concentration. The level of trichloroacetic acid is main-

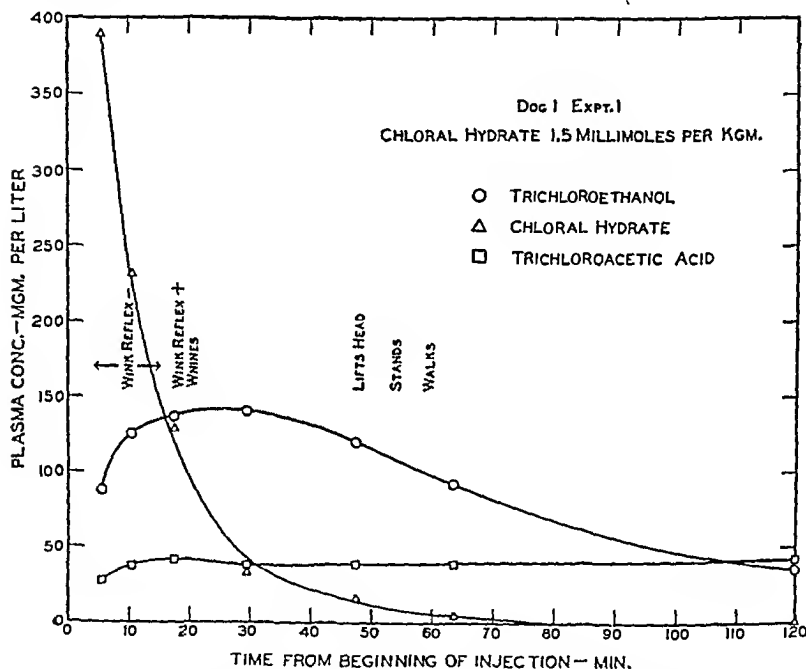


FIG. 1

Chloral hydrate, 1.5 millimoles (248 mgm.) per kgm., given intravenously over a period of 2½ min. to an 11 kgm. dog. The drug was given as a 10 per cent aqueous solution. All samples were taken from veins of the legs. Values for all three compounds were zero in a preinjection sample.

tained almost constant throughout the duration of the experiment. The persistence of trichloroacetic acid in the plasma of human subjects has previously been reported by Paykoç and Powell (25).

The plasma levels of trichloroacetic acid and trichloroethanol permit a rather accurate estimate of the amounts of these substances produced from chloral hydrate. Two dogs were given trichloroacetic acid (as the sodium salt) intravenously in doses of 6 and 10 mgm. per kgm., respectively. The plasma levels were such as would result if the substance had been distributed in 20 and

22 per cent of the body weight, respectively. This is in good agreement with the report of human experiments (25). If the trichloroacetic acid in the two dogs of figs. 1 and 4 were distributed in 20 per cent of their body weights, the total amounts of trichloroacetic acid calculated to be in the animals would correspond to 3 per cent and 4 per cent, respectively, of the doses of chloral hydrate.

An estimate of the yield of trichloroethanol from chloral hydrate can be arrived at by comparing the plasma levels produced by injecting into the same animals the molar equivalent doses of the alcohol itself. The results of these experiments are shown in figs. 2 and 3. Comparison of fig. 1 with fig. 2 shows that after 40 min. the levels of trichloroethanol scarcely differ by more than the ex-

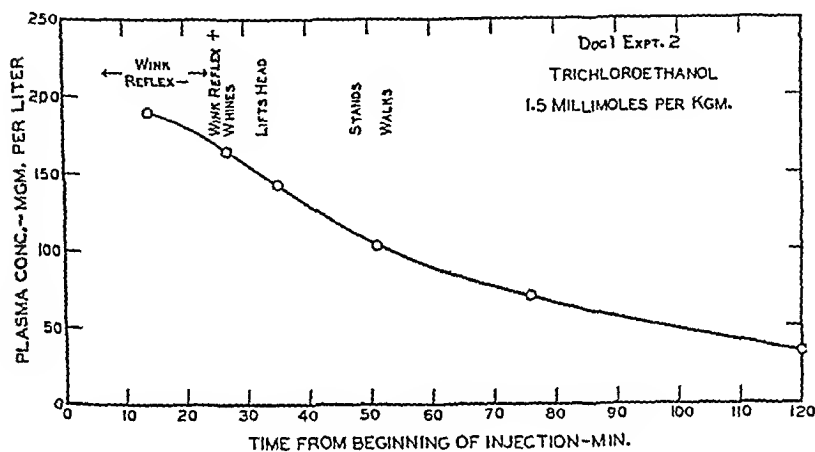


FIG. 2

Trichloroethanol, 1.5 millimoles (224 mgm.) per kgm., given intravenously at a decreasing rate over a period of 18 min. to the same dog as that of fig. 1. The experiment was performed 30 days after that of fig. 1. The drug was given as a 3 per cent aqueous solution. The first three samples shown were taken from the jugular veins, the later samples from veins of the legs. The concentrations shown are those of trichloroethanol. No chloral hydrate or trichloroacetic acid were found. Trichloroethanol was zero in a preinjection sample.

perimental error. In figs. 3 and 4 the correspondence is not so close until after 1 hr. Both of these pairs of experiments indicate that a very high proportion of the chloral hydrate, perhaps all of that not oxidized to the acid, is reduced to the alcohol.

In neither of the dogs receiving trichloroethanol did chloral hydrate or trichloroacetic acid appear in the plasma in concentrations measurable by the present methods. Simple oxidation is not a process of any physiological significance in the inactivation of trichloroethanol.

The course of the chemical reaction undergone by chloral hydrate can now be rather completely described. It is a very rapid reaction in which a small part is oxidized and most, perhaps all, of the remainder reduced. The removal of

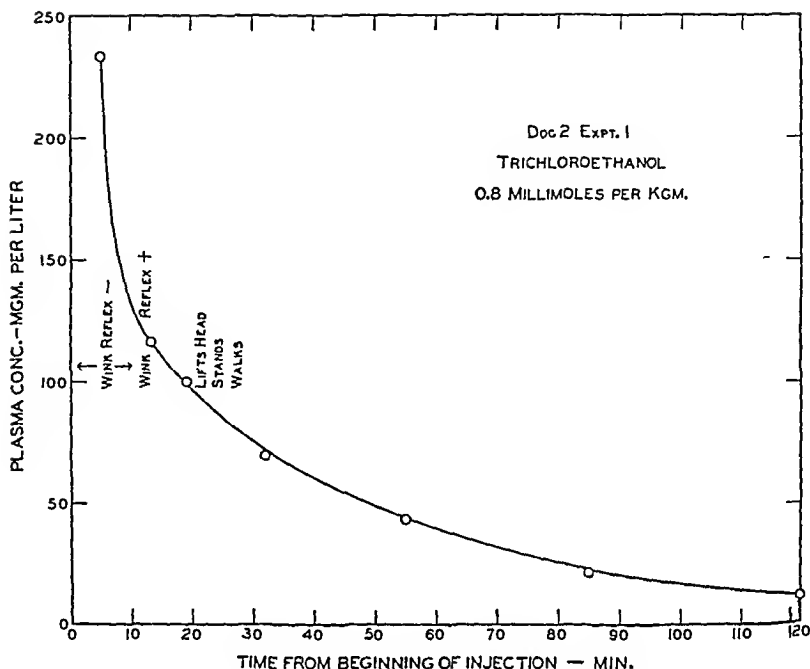


FIG. 3

Trichloroethanol, 0.8 millimoles (120 mgm.) per kgm., given intravenously over a period of $1\frac{1}{2}$ min. to an 18 kgm. dog. The drug was given as a 5 per cent aqueous solution. The first two samples shown were taken from the jugular veins, the later samples from veins of the legs. The concentrations shown are those of trichloroethanol. No chloral hydrate or trichloroacetic acid were found. Trichloroethanol was zero in a preinjection sample.

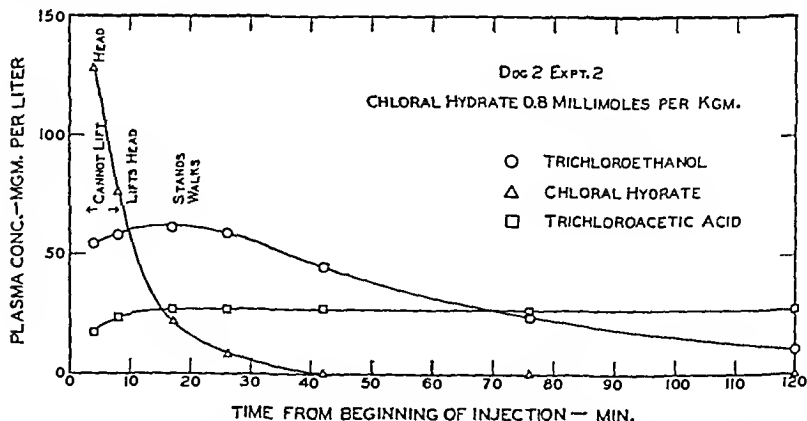


FIG. 4

Chloral hydrate, 0.8 millimoles (132 mgm.) per kgm., given intravenously over a period of $\frac{1}{2}$ min. to the same dog as that of fig. 3. The experiment was performed 5 days after that of fig. 3. The drug was given as a 12 per cent aqueous solution. The first two samples shown were taken from the jugular veins, the later samples from veins of the legs. Values for all three compounds were zero in a preinjection sample.

trichloroethanol from the plasma is relatively slow. Free trichloroethanol accordingly accumulates in considerable concentrations.

THE ROLE OF TRICHLOROETHANOL IN CHLORAL HYDRATE NARCOSIS. It is hardly conceivable that trichloroethanol could be present in the plasma in the concentrations found after the doses of chloral hydrate without exerting a significant effect on the central nervous system. Yet whether the trichloroethanol is sufficient to account for *all* of the narcotic phenomena is not a simple problem to settle. An approach toward a judgment of this question may be made by comparing the neurological effects produced in the same animal by equivalent molar doses of chloral hydrate and of trichloroethanol. A few gross neurological observations are indicated on the figures.

In both dogs, at the early stages of recovery the trichloroethanol concentrations are somewhat higher in the experiment in which the alcohol was given than in the experiment in which chloral hydrate was given. The greatest differences (in Dog 2) do not, however, exceed 50 per cent of the lower value. Only at the stage of recovery of the ability to walk by Dog 1 was the level almost identical in the two experiments. During the first few minutes after the administration of chloral hydrate, there is in both animals a definite lack of correlation between the neurological condition and the plasma concentration of trichloroethanol. Dog 1 was much more deeply depressed during the first 20 minutes while the alcohol concentration was rising than later when the concentration was falling at the same levels. Dog 2 was recovering rapidly during a period when the alcohol concentration was almost constant. Since the most striking discrepancies between the depth of anesthesia and the concentration of trichloroethanol are seen during the first few minutes while there is still an appreciable concentration of chloral hydrate in the plasma, it might be tempting to assume that chloral hydrate itself has some narcotic activity and that the rapid fall of chloral hydrate concentration causes a corresponding lightening of anesthesia. However, experiments of this type do not furnish sufficient evidence to justify such a conclusion. Particularly when the amount of a narcotic in the brain is rapidly changing, the neurological condition cannot be assumed to be determined simply by the amount of drug in the brain at the time; nor can the amount in the brain be assumed to be accurately reflected by the blood concentration. For example, some of the effects of ethyl alcohol are more intense when the blood concentration is rising than when it is falling at the same level (27). The results of the present experiments are not, then, necessarily inconsistent with the hypothesis that trichloroethanol is the only narcotic substance present. Nor, however, do they prove conclusively that chloral hydrate *per se* is absolutely devoid of narcotic activity; but they do indicate that the quantities of free trichloroethanol present are sufficient to account, in large part at least, for the depressant effects of chloral hydrate.

EVIDENCE FOR THE IDENTITY OF THE SUBSTANCES DETERMINED IN PLASMA. Since all the arguments and conclusions developed earlier in this paper depend upon a knowledge of the identity of the substances found in the plasma, it is important to examine critically the evidence bearing on the specificity of the measurements. The substances here determined as

"trichloroethanol", "chloral hydrate", and "trichloroacetic acid" not having been actually isolated, their identification must rest on certain properties sufficiently characteristic to exclude other substances that might conceivably be present.

The extraction procedure effectively separates trichloroethanol from the substances that would be expected to interfere; viz., chloral hydrate, trichloroacetic acid, and urochloralic acid. Some distribution coefficients of trichloroethanol and chloral hydrate are shown in table 1. The large differences make the separation of the two substances easy. If the distribution coefficients were the same for plasma as for water, it can be calculated that the final 4 ml. portion of pentane would contain 13 per cent of the trichloroethanol originally in the plasma sample, and only 0.009 per cent of the chloral hydrate. Trichloroacetic acid and urochloralic acid, being water-soluble acids that are largely dissociated at the pH of plasma, do not pass into the initial heptane extract.

Chloral hydrate added to a sample of plasma in a concentration of 1 gm. per l. gave a reading in the analytical procedure for trichloroethanol equivalent to that produced by 4 mg. per l. of trichloroethanol. Trichloroacetic acid added to plasma in concentrations as high as 1 gm. per l. was not detectable. That a conjugation product of trichloroethanol, presumably urochloralic acid, present in urine is not determined by the analytical method for trichloroethanol was demonstrated by the following experiment. Urine from a dog that

TABLE 1
*Some distribution coefficients of trichloroethanol and chloral hydrate**

SOLUTE	DISTRIBUTION COEFFICIENT			
	Benzene/Water	"Heptane"/Water	"Pentane"/Water	"Pentane"/Sodium Chloride Soln.†
Trichloroethanol	3.5	0.41	0.34	2.8
Chloral Hydrate	0.03	0.005	0.003	0.012

* Determined by analysis of one phase by procedures similar to those described under "Chemical Methods." Except for trichloroethanol in pentane, the aqueous phase was the one analyzed. The concentrations of solute in the aqueous phase were in all cases less than 0.2 mgm. per ml. The experiments were performed at room temperature (21-25°C.).

† Aqueous solution, 300 gm. per l.

had received chloral hydrate was adjusted to pH 8 and extracted with benzene until no trichloroethanol was detectable by the analytical method. After the extracted urine was subjected to acid hydrolysis and readjusted to pH 8, 100 mgm. per l. was determinable.

In order to learn more about the properties of the substance in plasma designated "trichloroethanol", the following experiments were performed. On one of the plasma samples from each of the two experiments with chloral hydrate (figs. 1 and 4) duplicate procedures were performed in which the sodium hydroxide was added immediately after the dichromate solution rather than after 1 hr. In these tubes almost no color developed. This shows that the substance must be oxidized to give the Fujiwara reaction. Another dog was given an oral dose of 1 gm. per kgm. of chloral hydrate. Two hours later, during deep anesthesia, the plasma was found to contain 250 mgm. per l. of "trichloroethanol". A larger sample of this plasma was extracted in the same way as in the standard analytical procedure. The aqueous extract of heptane was divided into aliquot parts, some of which were shaken with heptane or with benzene before being salted and extracted with pentane. From the diminution of the intensity of color in the final Fujiwara reactions, the amounts of the color-producing substance that had been removed from water by the solvents, and thus its distribution coefficients, were calculated. The distribution coefficients found were: benzene/

water, 3.2; heptane/water, 0.35. These distribution coefficients scarcely differ by more than the experimental error from those determined with authentic trichloroethanol (table 1).

The characterizing properties of the three substances found in blood after the administration of chloral hydrate may be summarized as follows:

"Trichloroethanol." It is extracted by a procedure that excludes chloral hydrate, trichloroacetic acid, and urochloralic acid. It does not itself give the Fujiwara reaction, but on oxidation with dichromate yields a product that gives a crimson color. This excludes chloral hydrate, trichloroacetic acid, and chloroform. Its distribution coefficients between benzene and water and between heptane and water are close to those of trichloroethanol. The "trichloroethanol" produced from chloral hydrate disappears from the plasma at very nearly the same rate as that present after the injection of trichloroethanol itself.

"Chloral Hydrate." It gives a crimson color directly in the Fujiwara reaction. It is not extracted from plasma by heptane. In strongly alkaline solution at room temperature it rapidly decomposes in such a way that extraction with heptane removes from the aqueous phase all of the material giving the Fujiwara reaction.

"Trichloroacetic Acid." It gives a crimson color directly in the Fujiwara reaction. It is not extracted from plasma by heptane. In strongly alkaline solution at room temperature it does not decompose to give chloroform. The "trichloroacetic acid" produced from chloral hydrate persists in the plasma at an almost constant level, as does injected trichloroacetic acid.

SUMMARY

Methods are described for the determination of trichloroethanol, chloral hydrate, and trichloroacetic acid when present together in plasma.

When chloral hydrate is administered intravenously to dogs, the concentration of chloral hydrate in the plasma falls rapidly. Trichloroethanol and trichloroacetic acid appear very quickly in the plasma and reach such concentrations as to indicate that only a small part of the chloral hydrate is oxidized and that a high proportion, perhaps all, of the remainder is reduced to the alcohol. The removal of trichloroethanol from the plasma is relatively slow, and the concentrations reached are sufficient to account, in large part at least, for the depressant effects that follow the administration of chloral hydrate.

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THE EFFECTS OF ANALGESIC DRUGS UPON EXCISED FROG AND TERRAPIN HEARTS AND UPON THE TERRAPIN CARDIAC VAGUS NERVE¹

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In a previous investigation we were able to show that 1-amino-1-phthalidylpropane hydrochloride produced a slowing of the heart rate and a decrease in the force of the contractions in both anesthetized and decerebrated cats and dogs. Aminopyrine, on the other hand, had no marked effect upon the force of the contractions but caused, in most instances, an acceleration in the rate of the heart beat. We thought it would be interesting to determine the effects of these and other analgesic drugs upon the excised frog and terrapin hearts and study their effect upon the cardiac vagus nerve of the terrapin.

METHOD Excised hearts of large frogs of the species *Rana pipiens* were used. The method of experimentation was the same as that previously described (2). Thirty-five terrapin of the species *Chrysemys marginata* also were used. The method employed being the same as that described previously by one of us (3).

The drugs experimented with were racemates A and B of 1-amino-1-phthalidylpropane hydrochloride, aminopyrine, antipyrine and acetanilid. These were dissolved in Ringer's solution freshly made up each day.

RESULTS *Frog Heart* Both racemates A and B of 1-amino-1-phthalidylpropane hydrochloride have greater cardiac depressant actions upon the excised frog's heart than have other analgesics such as aminopyrine, antipyrine and acetanilid. In the 53 experiments performed with either antipyrine or aminopyrine the results were variable. With $N/200$ and $M/100$ dilutions a temporary increase in both the rate and the height of contractions were noted followed by a decrease in the rate with no change in the height of the contractions. In table 1, are the collected results of 126 experiments performed upon 32 frog hearts in which four different molecular concentrations of racemates A and B of 1-amino-1-phthalidylpropane hydrochloride were used. With the $M/100$ solutions 50 per cent or more of the hearts were stopped in diastole.

In these experiments as in those on dogs, cats and rabbits, racemate A appears to be approximately twice as depressant as racemate B (See table I). The results in figure 1 are typical of all experiments performed. In this figure at 1, in the upper curve, the heart was perfused with an $N/200$ solution of racemate A and in the lower curve with racemate B. At 2 in the upper curve an $M/100$ solution of racemate A was used and in the lower curve racemate B was employed. It will be noted that the depression of the heart caused by racemate B in $N/100$ solution (at 2) is approximately equal to that caused by racemate A in $N/200$ solution, (at 1).

Turtle Heart Although the analgesic drugs studied had no effect upon the height of the contractions of the terrapin heart, they did, however, slow its rate.

¹ This research was made possible through a grant by Smith, Kline and French Laboratories.

In eight animals in which racemate A was applied to the heart in concentrations of M/500, M/250 and M/100 the average rates of contractions were diminished by 20, 30 and 43 per cent respectively. In the seven animals in which racemate B was used in concentrations of M/250, M/100 and M/50 the average rates were decreased by 20, 35 and 40 per cent respectively. On the terrapin heart as

TABLE 1

A table showing the effects of racemates A and B of 1-amino-1-phthalidylpropane hydrochloride, upon the height and the rate of contraction of the excised frog's heart

One hundred twenty-six experiments were performed upon 32 hearts. — indicates no effect; — + indicates a reduction of less than 50 per cent; + — a reduction of 50 per cent or more; + complete stoppage of the heart.

DRUG	M/100			M/300				M/200				M/100				NUMBER OF EXPERIMENTS
	—	— +	— +	—	— +	— +	+	—	— +	— +	+	—	— +	— +	+	
Height																
Racemate A	1	2	3		2	5	4		1	16	8		4	18		64
Racemate B	2	5	1	5	1	3		1	8	11	3		11	11		62
Rate																
Racemate A	1	2	3		4	3	4		4	13	8		4	18		64
Racemate B	3	5		3	4	2		2	8	10	3	1	10	11		62

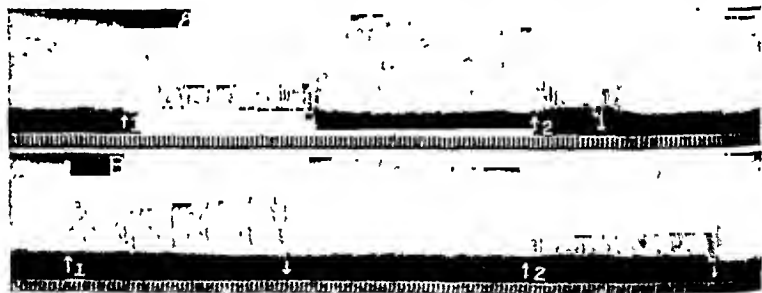


FIG. 1

In each record the upper curve indicates the contractions of the excised ventricle of the frog (*Rana pipiens*) and below it is the time in intervals of 10 seconds. The up stroke in each curve was made by the systolic contraction and the down stroke by the diastolic. Between the arrows at 1 the Ringer's solution was removed and replaced by an M/200 solution of the drug. In the upper record (A) racemate A, and in the lower (B) racemate B of 1-amino-1-phthalidylpropane hydrochloride were used. Between the arrows at 2, M/100 solutions of the same drugs were substituted for the Ringer's solution.

on the frog heart racemate A appears to be twice as toxic as racemate B. In the few experiments performed with M/250, M/100 and M/50 concentrations of acetanilid the average cardiac rates were decreased by 10, 22 and 25 per cent respectively. In as much as such concentrations may block the response of the heart to stimulation of the vagus, it would appear that these drugs act directly on cardiac muscle or on the Sinoauricular node.

TABLE 2

In this table are assembled all of the data on the experiments in which analgesic drugs were used in depressing the cardiac vagus nerve in the terrapin

— Cessation of heart beat upon vagus stimulation as in control. —+ Vagus nerve depressed less than 50 per cent as indicated by the number of heart beats during nerve excitation. +- Vagus nerve depressed 50 per cent or over. + Complete block of the cardiac vagus nerve as indicated by no change in the ventricular rate during excitation of the nerve.

ANALGETIC	NUMBER OF EXPERIMENTS	M/500			M/250				M/100				M/50			
		+-	-+	-	+	+-	-+	-	+	+-	-+	-	+	+-	-+	-
Racemate A	60	3	13	7	1	9	6	2	8	5	1	1	2	1	1	
Racemate B	59		4	6			8	9		2	10	4	6	6	3	1
Aminopyrine	45	2	7	3	1	4	5	3	1	4	6		1	6	2	
Acetanilid	27		1	2			5	3			6	3		4	3	
Antipyrine	38		2	2			4	7			4	7		3	8	1

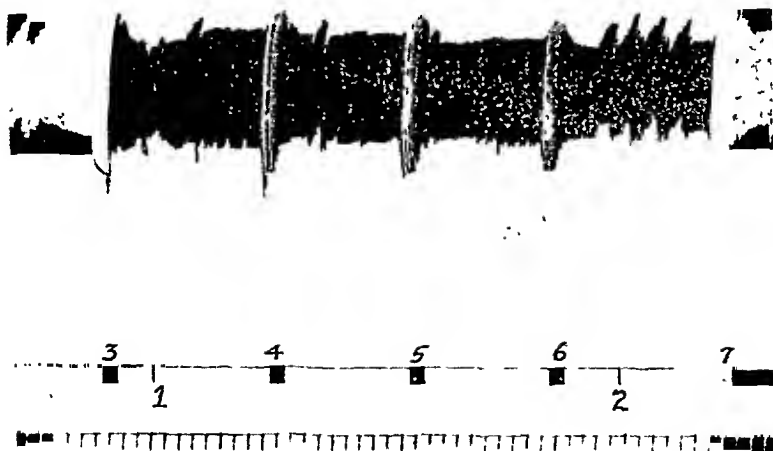


FIG. 2

Top record is that of the contractions of the ventricle of the terrapin *Chrysemys marginata*. The up stroke is systole and the down stroke diastole. The bottom record is the time in intervals of 20 seconds and above it a record of the duration of stimulation of the right vagus nerve

1. Ringer's solution which was bathing the ventricle was replaced by a solution of Ringer-racemate B of 1-amino-1-phthalidylpropane hydrochloride M/100.

2. Racemate B solution replaced by Ringer's solution.

3 to 7 inclusive — Times and durations of stimulation of the right vagus nerve. During vagal stimulation the ventricle contracted four times in 4 and 5 and five times in 6. The control ventricular rate was 75 contractions per minute which was decreased to 60 contractions per minute while the drug was being applied to the heart.

Cardiac Vagus Nerve. All of the analgesic drugs which we studied, either partially or completely blocked the response of the heart to cardiac vagus nerve stimulation, table 2. Aminopyrine and racemate A appeared to be the most

active of the group, and antipyrine, the least. Again in these studies, racemate A was found to be approximately twice as toxic as racemate B.

Figure 2 is presented as being typical of all such experiments. In this figure, at 1, M/100 solution of racemate B (in Ringer's solution) was substituted for the normal solution bathing the heart and at 2 the racemate B Ringer's solution was replaced by Ringer's, the bath being changed four times before the vagus nerve was excited electrically. At 3, the right vagus nerve was stimulated electrically before the application of the drug and again at 7 after removal of the solution containing the drug. At 4, 5 and 6 the right vagus nerve was stimulated while the drug was bathing the heart. Upon examination of the record four escapes of the heart from vagal inhibition will be seen in 4 and in 5, and 5 escapes in 6 during electrical excitation of the vagus nerve. The same concentration of racemate A applied to the heart caused complete blocking of the response of the heart to vagus nerve excitation in 8 out of 15 experiments and M/50 of racemate B caused complete block of the response to vagus nerve stimulation in 6 of the 14 experiments performed.

SUMMARY

1. Racemates A and B of 1-amino-1-phthalidylpropane hydrochloride when applied to the perfused excised frog heart, decrease the rate and force of the cardiac contractions. These changes are similar to those seen in the intact mammalian heart upon intravenous injection of these compounds. The results with aminopyrine and antipyrine were variable.

2. Racemates A and B when applied to the exposed terrapin heart decrease the rate but not the height of the contractions.

3. Racemates A and B of 1-amino-1-phthalidylpropane hydrochloride, aminopyrine, acetanilid and antipyrine decrease the response of the heart to cardiac vagus nerve excitation. In high concentrations, racemates A and B and aminopyrine may cause complete block of the response of the heart to vagus nerve stimulation.

4. Racemate A of 1-amino-1-phthalidylpropane hydrochloride was found to be twice as toxic to the frog and turtle heart as was racemate B.

5. All of the analgesic drugs studied have a direct depressant action upon cardiac muscle.

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THE INFLUENCE OF ATROPINE AND SCOPOLAMINE ON THE CENTRAL EFFECTS OF DFP

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I. INTRODUCTION. Di-isopropyl fluorophosphate (DFP) has been shown to inactivate irreversibly, within certain limits, the cholinesterases in the animal organism. To the present time no other major action has been shown for DFP and its toxic properties have been attributed to this phenomenon; it has been assumed that the destruction of cholinesterase (ChE) allows for the building up of concentrations of acetylcholine (ACh) in the central nervous system and in the periphery which eventually prove lethal.

It has been reported previously (1) that in cats, atropine is an effective prophylactic and therapeutic agent against DFP; it annuls both the muscarinic effects and the central excitatory action of DFP. Our experiments were planned to dissect this protective action and to obtain, in particular, data concerning the central effects of atropine. It appeared that DFP provided an excellent tool for studying the effects of increased concentrations of endogenous ACh in the central nervous system and for determining the ability of atropine to alter these effects.

The body of this report concerns experimental work and extensions of it which were previously reported in abstract form (2).

II. METHODS. Experiments were performed on cats and monkeys. Under local (2% procaine) anesthesia the trachea was cannulated, the calvarium was laid bare, and positive-pressure artificial respiration was begun. The animal was curarized to prevent the appearance of muscle potentials in the electroencephalogram, the approximate dosage required for complete curarization was two to three units per kilogram body weight of intocostrin (Squibb). Electrical potentials were led off the cortex by means of 3 screw electrodes inserted into the calvarium, these were placed over the right frontal, left frontal, and right occipital areas as illustrated in figure 1. Each active electrode was pitted against an indifferent electrode, usually placed on the left ear, which also served as a common ground. The electrodes were led into a four-channel Grass ink writing electroencephalograph. Simultaneous electrocardiograms were recorded on the same instrument using the standard second lead.

All solutions were made up in distilled water. With the exception of the atropine and scopolamine all solutions were made fresh daily and used within three hours.

III. EXPERIMENTAL. a. *Effect of Curare*. All of the cats and monkeys which were used were completely curarized. In none of our experiments, involving 31 animals, did we observe any effects on the EEG which could be attributed to curare. No change occurred in the electrical activity of the brain when the peripheral effect of curare was wearing off and no change ensued when repeated

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doses of curare were given to such animals to maintain complete muscular relaxation. In so far as it may be concluded from the electroencephalograms it appears that in the dose range we employed, curare has no central effect.

b. *Effect of Atropine.* The effect of atropine on the control EEG was tested in 3 cats and 1 monkey. The control EEG in cats consisted mostly of moderate voltage waves in frequencies of 12-18 per second; following the injection of atropine sulphate (1 mgm. intravenously)¹ there was a shift in the pattern of the EEG toward the high voltage slower waves with frequencies of 6-10 per second being the most usual. Repeated injections of the same amount of atropine caused further depression in the frequency pattern and the majority of waves fell in the 3-8 per second range. It was apparent that in these curarized cats atropine affected the control EEG and produced considerable slowing. How-

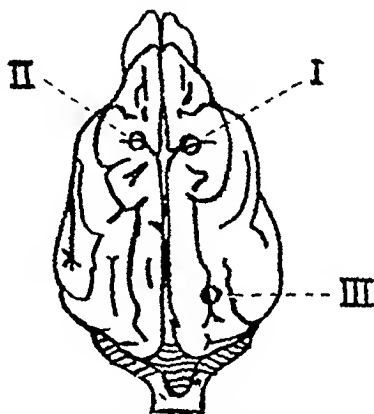


FIG. 1. SCHEMATIC REPRESENTATION OF ELECTRODE PLACEMENT

The points designated I, II, and III represent the approximate location of screw electrodes in the right frontal (RF), left frontal (LF), and right occipital (RO) areas.

ever, it should be emphasized that 1 mgm. atropine sulphate causes no central nervous system symptoms in cats.

Similar EEG results were obtained in the monkey.

c. *Effect of Scopolamine.* The effect of scopolamine on the control EEG was tested in 3 cats and 1 monkey. The intravenous injection of 1 mgm. scopolamine produced an effect similar to that of atropine, namely, an increase in voltage and a decrease in frequency. After the injection of 5 mgm. scopolamine in divided doses the frequency fell from a range of 16-24 per second to 8-12 per second.

These effects of atropine and scopolamine are illustrated in figures 2 and 3.

d. *Effect of DFP.* The effect of DFP on the EEG was tested in 15 cats and 3 monkeys. In most cases the initial injection of DFP was 3 mgm., this being the LD₁₀₀ for our sample of the drug. Within one minute following injection

¹ In this report all doses are expressed as mgm. per kgm. of body weight.

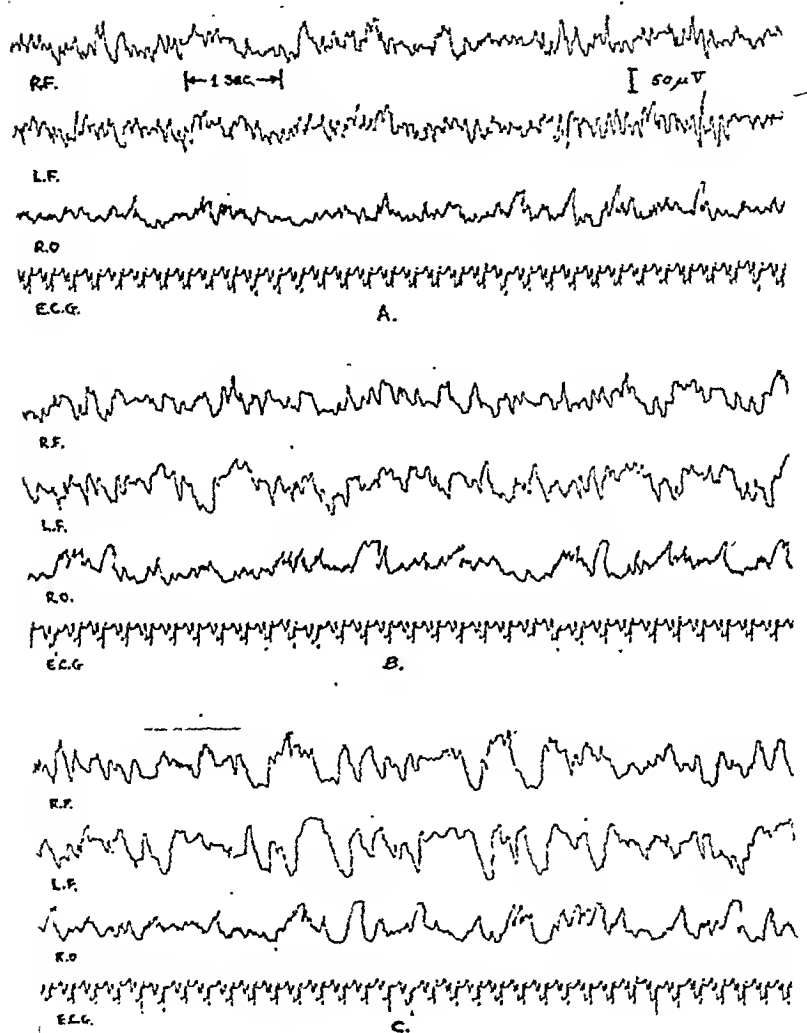


FIG. 2. EFFECT OF ATROPINE ON THE EEG

A. Control tracing from an unanesthetized, curarized cat. B. Tracing obtained 2 minutes after the intravenous injection of 1 mgm. of atropine sulphate. C. Tracing obtained 1 minute after the intravenous injection of an additional 1 mgm. of atropine sulphate.

the first effects were noted; in the great majority of cases these consisted of an increase in the frequency and a decrease in the voltage. The picture consisted of very high frequency (24-40 per second) and low voltage waves which persisted

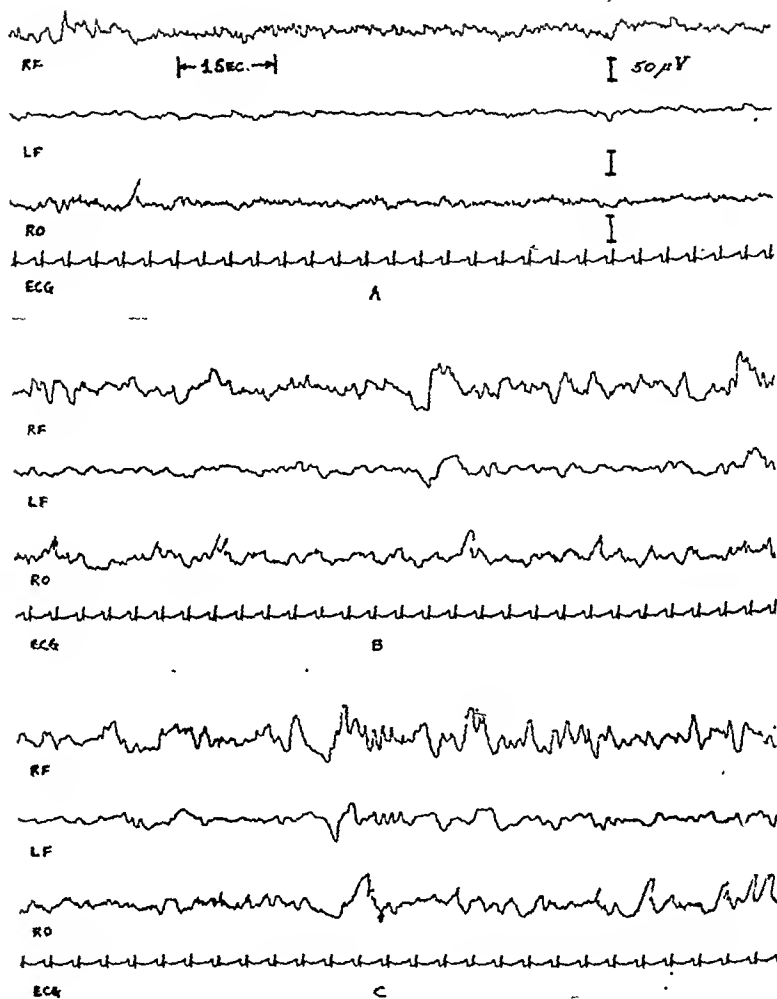


FIG. 3. EFFECT OF SCOPOLAMINE ON THE EEG

A. Control tracing from an unanesthetized, curarized cat. B. Tracing obtained 2 minutes after the intravenous injection of 1 mgm. of scopolamine hydrobromide. C. Tracing obtained 30 seconds after the intravenous injection of an additional 2 mgm. of scopolamine hydrobromide.

until the experiment was terminated or until atropine was administered. In an occasional experiment, the increased frequency was accompanied by an increase in voltage.

In several instances the DFP was administered in small divided doses; under these circumstances, the same increase in frequency was noted but it was not until a total dose of 3 mgm. had been given that a frankly convulsant picture

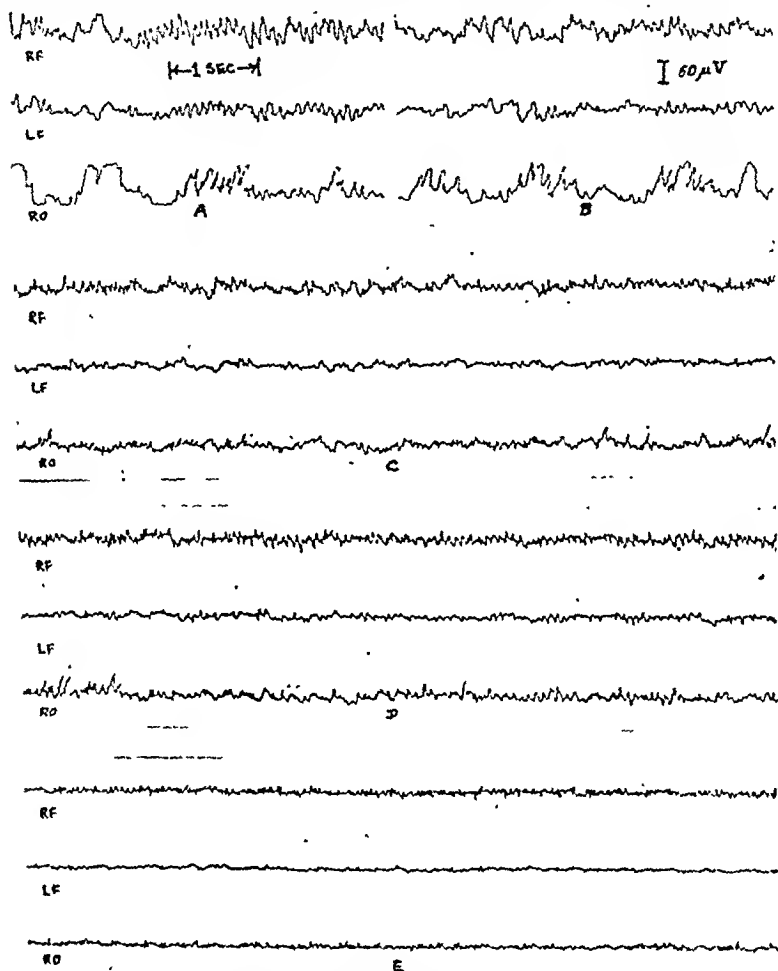


FIG 4 THE EFFECT OF REPEATED INJECTIONS OF DFP

A. Control tracing from an unanesthetized, curarized cat. B. Tracing obtained 2 minutes after the first injection of 1 mgm. of DFP. C. Tracing obtained 1 minute after the second injection of 1 mgm. of DFP. D. Tracing obtained 5 minutes after the third injection of 1 mgm. of DFP. E. Tracing obtained after a total of 10 mgm. of DFP.

appeared. With further increments of the drug above this level, the frequencies became extremely high, but in no instance in doses up to 15 mgm. were we able to show complete suppression of cortical activity.

An illustration of the action of DFP in the cat is shown in figure 4.

e. *The Abolition of the Central Effect of DFP by Atropine and Scopolamine.* The effect of the belladonna alkaloids was investigated after the DFP pattern had become established to varying degrees in the EEG. The intravenous injection of 3 mgm. of atropine following the 3 mgm. dose of DFP resulted, within one minute, in a return of the EEG pattern to its control level of activity; this was also true following the injection of 3 mgm. of scopolamine. Injections of 1 or 2 mgm. of either drug returned the pattern toward control activity but further doses were often necessary to prevent the recurrence of DFP action.

In the animals which had received larger amounts of DFP, comparably larger amounts of atropine and scopolamine were necessary to return the EEG pattern to its control level and to hold it there.

An illustration of the annulment of the convulsant activity of DFP by atropine is shown in figure 5.

f. *Prophylactic Action of Atropine and Scopolamine.* The ability of previously injected atropine or scopolamine to prevent the appearance of cortical changes following DFP was observed in 3 cats and 2 monkeys. In each instance the prior administration of 3 mgm. of atropine or 3 mgm. of scopolamine prevented any changes in frequency or voltage following the injection of 3 mgm. of DFP. It was only after the administration of 5 to 6 mgm. of DFP that any changes were noted; these changes were limited to small increases in frequency, but the true convulsant pattern did not appear during the limits of our experiments. These observations confirm the clinical results (1).

As a direct corollary to these experiments we were able to show that the intravenous injection of atropine which abolished the established central effect of DFP served further to prevent the action of subsequently administered DFP in doses up to 6 mgm.

g. *Contrast with Other Convulsant Drugs.* To emphasize the fact that this preventing or annulling action of atropine was specific for DFP, experiments were carried out on 12 cats using pentamethylenetetrazol (Metrazol), strychnine, and gammexane (the γ isomer of hexachlorocyclohexane). In none of these animals was atropine able to prevent or abolish the convulsant activity produced by intravenous doses of 30 mgm. of metrazol, 2 mgm. of strychnine, or 4 mgm. of gammexane.

It should be emphasized that the convulsant picture evoked by these three drugs differs in rhythm, amplitude and pattern from that of DFP. Whereas DFP characteristically produces an extremely high frequency (24-40 per second), low voltage EEG which persists unaltered for hours, these other drugs elicit short bursts of activity characterized by high voltage, spiking waves in a predominant frequency range of 12 to 18 per second which alternate with periods of absolute electrical quiescence. An illustration of this difference between DFP and metrazol is shown in figure 6.

IV. DISCUSSION. The observations on curarized animals which failed to reveal any effect of the drug on the EEG are in contrast to those of Pick and Unna (3) who reported that curare alkaloids depressed cortical potentials in the frog.

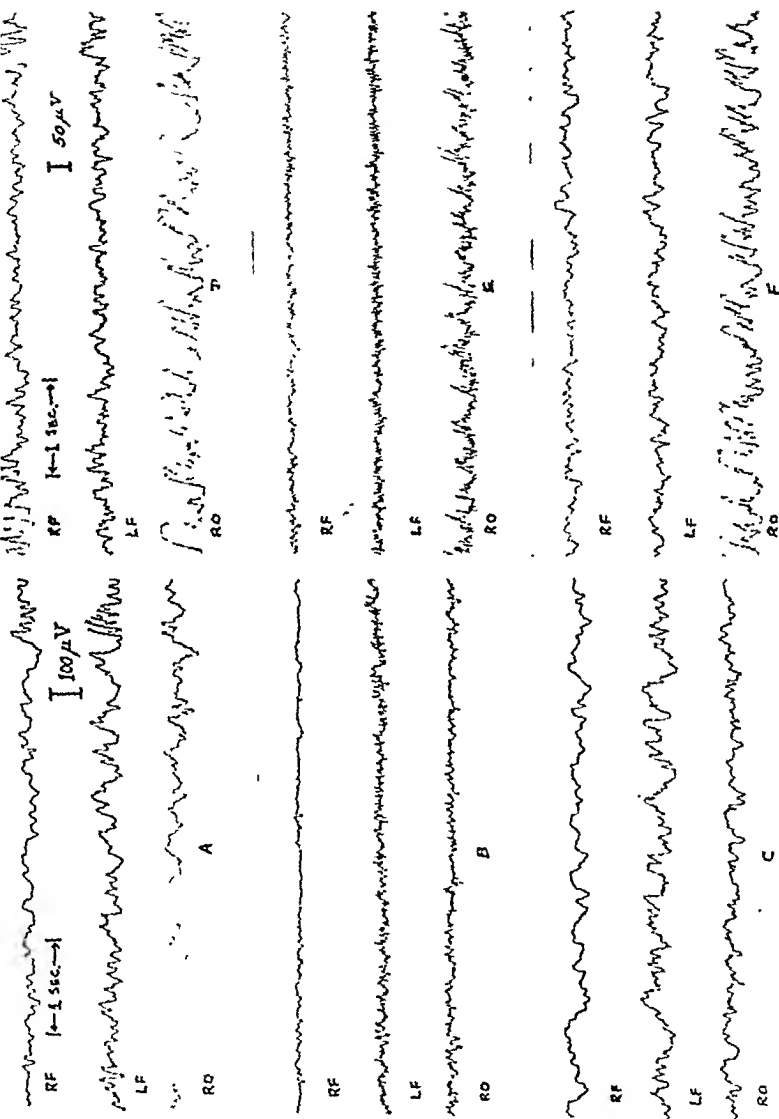


FIG. 5. THE ANTI-DFP EFFECT OF ATROPINE AND SCOPOLAMINE

A. Control tracing from an unanesthetized, curarized cat. B. Tracing obtained 1 minute after the intravenous injection of 3 mgm. of DFP. C. Tracing obtained 1 minute after the injection of 2 mgm. of atropine sulphate and 1 minutes after B, showing a return to control activity. D. Control tracing from an unanesthetized, curarized cat. E. Tracing obtained 1 minute after the injection of 3 mgm. of DFP. F. Tracing obtained 1 minute after the injection of 3 mgm. of scopolamine hydrobromide and 4 minutes after E, showing a return to control activity.

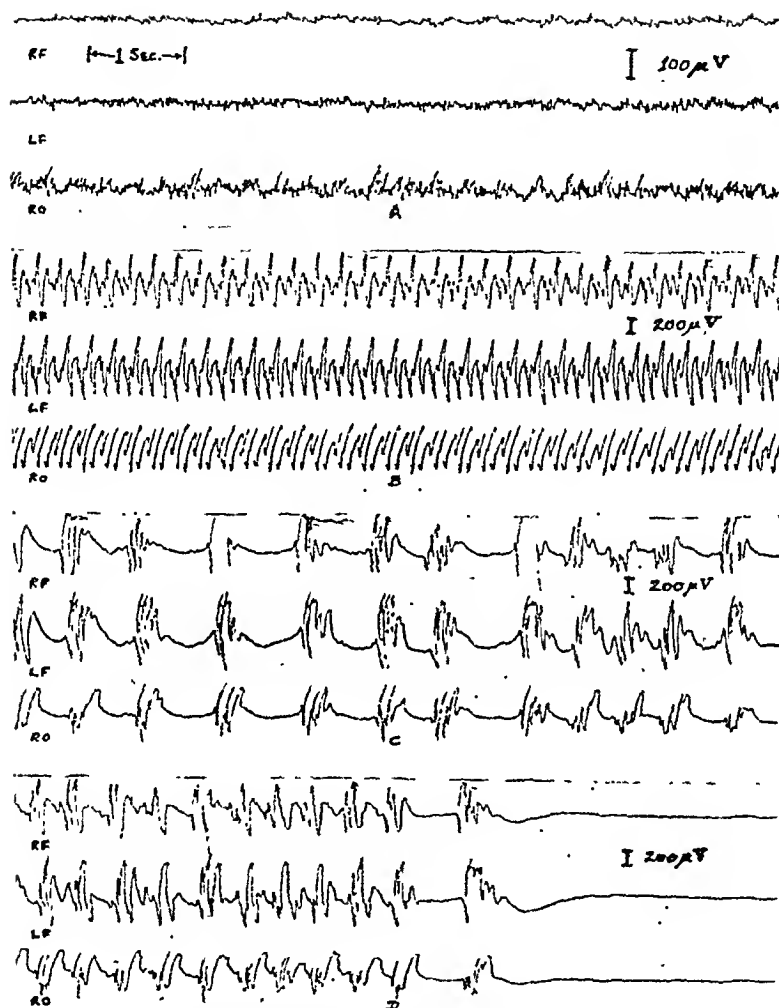


FIG. 6. CONTRAST BETWEEN DFP AND METRAZOL

A. Rapid frequency, low voltage EEG obtained after the injection of 3 mgm. of DFP. B, C, and D. Convulsant pattern following the injection of 30 mgm. of Metrazol. These latter records are from a different animal. Note the difference in calibration.

On the other hand, they are in direct confirmation of the experiments of Smith et al. (4) who showed that, in the human, d-tubocurarine had no cortical, analgesic, or anesthetic effect.

An effect of atropine on the EEG was reported by Darrow and his co-workers

(5) in 1944; they demonstrated that atropine increased the number of high potential EEG waves in cats treated with beta erythroidin. In a later publication (6) dealing with human experiments, however, Darrow's group was unable to confirm this action completely on non-curarized subjects. Our experiments definitely showed a potent effect of atropine and scopolamine on the EEG of normal animals and the change produced was similar to that which Darrow had reported. We were unable to obtain tracings from non-curarized animals and it was not possible, therefore, to determine whether this atropine effect is unalterably bound up with curarization.

Opinion remains divided concerning the ability of atropine to annul the central nervous system actions of acetylcholine. The observation by Sjostrand (7) that ACh first stimulates and then depresses the electrical activity of the cortex gave impetus to many experiments and a considerable bibliography on the subject has grown in the past ten years. Miller, Stavsky, and Woonton (8) were able to show that intravenous atropine prevented the appearance of large, fast spikes produced in the electrocorticogram by the local application of ACh to the eserized cortex. Chatfield and Dempsey (9) reported that atropine did not affect the spontaneous activity of the cortex but did abolish some of the electrical effects produced by the local application of ACh to the cortex previously treated with the anti-cholinesterase, prostigmine. In contrast to this, Brenner and Merritt (10) reported that intravenous atropine did not abolish the electrical changes produced by direct application of ACh to the cortex, but that atropine directly applied to the cortex did alter the spontaneous activity. Bülbring and Burn (11), Gesell and Hansen (12), and Calma and Wright (13) showed that atropine exerted a powerful anti-ACh action in the central nervous system. Finally, Bornstein (14) observed that intracisternal ACh produced first an increase in frequency and amplitude in the EEG and then a flattening; these effects he was able to prevent or abolish with atropine given intravenously.

The major toxic action of DFP has been shown to be the irreversible inactivation of ChE; by inference, then, the toxic manifestations of DFP may result from a steady accumulation of ACh. Our experiments indicate conclusively that DFP produces an increase in frequency in the EEG such as has been described for intracisternally and topically applied ACh. In addition, this change in the electrical pattern of the brain could be prevented or annulled by the intravenous administration of adequate amounts of atropine or scopolamine. We believe that these facts lend support to the hypothesis that ACh may be involved in central transmission and that atropine exerts its central action by preventing ACh effects. The fact that atropine in the doses we have used does not abolish all activity does not necessarily invalidate the hypothesis; indeed, it is highly provocative, in support of the hypothesis, that the EEG of normal animals is altered by such doses of atropine.

The conclusion concerning the antagonism of atropine toward the central actions of ACh is fortified in a measure by reason of the fact that large doses of atropine exerted no protective action against the central convulsions of strychnine, pentamethylenetetrazol (Metrazol), or gammexane, drugs which pre-

sumably are convulsant through mechanisms differing from that of ACh. Our results do not necessarily conflict with the findings of Longino and Preston (15) who reported that atropine in very large doses protected against the lethal action of strychnine in the mouse.

Our results with atropine are not in agreement with those of Heymans and Jacob (16) who reported that atropine did not control the convulsions produced by DFP. However, since our original communication (2) appeared, Grob, et al. (17) have reported that atropine decreased the spontaneous activity in the human electroencephalogram and resulted in a return to normal of the human EEG which had been stimulated by DFP.

SUMMARY

1. In cats and monkeys intravenous intocostrin (Squibb) in doses sufficient to produce complete skeletal and respiratory paralysis did not affect the EEG pattern.

2. Intravenous atropine and scopolamine produced a change in the control EEG characterized by a decrease in frequency and an increase in voltage.

3. Intravenous DFP produced a constant EEG effect characterized by an increase in frequency and a decrease in voltage.

4. The action of DFP could be prevented or abolished by the intravenous administration of atropine or scopolamine.

5. Atropine and scopolamine had no prophylactic or therapeutic effect on the convulsions caused by pentamethylenetetrazol (Metrazol), strychnine, or gammexane.

6. These data indicate that atropine prevents or annuls the central actions of ACh.

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THE INFLUENCE OF SULFHYDRYL COMPOUNDS ON DIURESIS AND RENAL AND CARDIAC CIRCULATORY CHANGES CAUSED BY MERSALYL¹

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It has been shown by Long and Farah (1, 2) that the thiol compounds cysteine, glutathione, and 2,3 dimercaptopropanol (BAL) markedly reduce the cardiac toxicity of the mercurial diuretic mersalyl. These findings have been confirmed by Lehman (3) who used thioglycolic acid. Since the chief pharmacological action of mersalyl is on the kidney it was of interest to study the influence of some sulfhydryl compounds on the diuretic action of mersalyl, and to find out whether abolishing of cardiac toxicity can only be obtained at the expense of abolition of kidney action.

METHODS: Diuresis experiments were performed on 16 anesthetized and 60 unanesthetized rabbits, and on 29 anesthetized dogs

The anesthetized rabbits weighed between 1.8 and 2.2 kgm. On the day before the experiment they were given by stomach tube 25 cc. of tap water and 0.2 grams of ammonium chloride per kgm. of body weight. Urethane was given intravenously in a dose of 0.8 to 1.0 gram per kgm. The bladder was cannulated through an incision in the lower abdomen and blood pressure was recorded through a cannula inserted into the right jugular vein. The intramuscular injections were given into the posterior aspect of a hind leg. Urine was collected in graduated cylinders at 5 minute intervals. After an adequate control period the diuretic was injected intravenously and urine was collected for 90 to 120 minutes following the injection. In some of the animals a 2 per cent solution of BAL in saline was given either intramuscularly or intravenously in a dose of 10 mgm. per kgm. immediately following the injection of diuretic.

The unanesthetized rabbits weighed 1.8 to 2.4 kgm. and were pretreated with water and ammonium chloride like the anesthetized rabbits. After emptying the bladder, the animals were placed into metabolism cages in groups of two; urine excretion was recorded in 30 minute periods for 5 hours. Since the rabbit does not readily empty its bladder spontaneously the urine was expressed by massaging the lower abdomen. In all experiments on unanesthetized rabbits total 5 hour excretion of chloride ion was estimated by the method of Van Slyke and Sendroy (4). All injections were given into a marginal ear vein and together amounted to a volume not exceeding 2 cc. per animal.

The dogs weighed 6.5 to 14.8 kgm. and were given by stomach tube 40 to 50 cc. of tap water per kgm. on the day prior to the experiment. The same amount of water was given 2 to 3 hours before the experiment was started. Anesthesia was made by giving intraperitoneally 0.7 cc. of dual urethane solution³ per kgm. Arterial and venous pressures and the electrocardiogram were recorded by methods described previously (2). Urine flow was measured by cannulating the ureters and recording the out-flow by means of a drop recorder and signal magnet.

¹ Supported by grants from the Schering Corporation, Bloomfield, New Jersey, and from the Wellcome Research Laboratories, Tuckahoe, New York.

² Wellcome Research Fellow in Pharmacology.

³ Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

In 6 experiments in anesthetized dogs, kidney volume was recorded by means of a kidney oncometer connected to a Marey tambour. Blood pressure and urine flow were recorded simultaneously with kidney volume.

Blood flow through kidney and hind leg was measured by means of a bubble flow meter (5) in heparinized dogs. Pretreatment with water and anesthesia were the same as described above. For kidney blood flow the bubble flow meter was connected to the proximal end of the left common carotid and to the distal end of the left renal artery. For blood flow through the hind leg the femoral artery was used. Following the attainment of constant blood flow levels the substances to be tested were injected either intravenously or intraarterially. Heparin⁴ in an initial dose of 3 mgm per kgm followed by 0.5 mgm per kgm. every half hour was given intravenously.

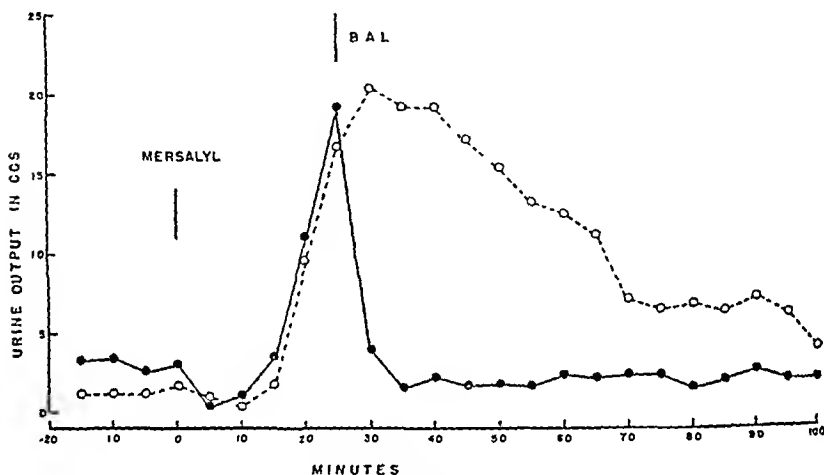


FIG 1 THE EFFECT OF BAL ON MERSALYL INDUCED DIURESIS IN THE ANESTHETIZED RABBIT

Male rabbit, 2.2 kgm. Anesthesia, urethane, 1.0 gram per kgm intravenously. Urinary bladder cannulated. Abscissa—time in minutes. Ordinate—cc of urine excreted in 5 minutes. At 0 time, 10 mgm per kgm of mersalyl was given intravenously. ○---○ first injection of mersalyl. ●—● second injection of mersalyl. BAL—5 mgm per kgm. of BAL was given intravenously 25 minutes after the administration of the second dose of mersalyl.

Mersalyl solutions were made up freshly by adding enough sodium hydroxide to dissolve salyrganic acid,⁵ cysteine, glutathione, and BAL were dissolved in 0.9% sodium chloride solution, all solutions were adjusted to pH 7.0 to 7.5 before injection. All doses of cysteine stated in mgm refer to cysteine hydrochloride.

RESULTS 1. *The effect of 2,3 dimercaptopropanol on the diuretic action of mersalyl.* In rabbits anesthetized with urethane, 10 mgm. per kgm of mersalyl given intravenously produced first a reduction in urine flow lasting 5 to 10 minutes. This was followed by an increase in urine flow, the maximum being reached within 20 to 30 minutes. Within 90 to 120 minutes urine flow returned

⁴ Kindly supplied by Eli Lilly and Company, Indianapolis, Indiana

⁵ Kindly supplied by Winthrop Chemical Company, Inc., New York, New York.

to approximately normal levels. If BAL was given at the peak of mersalyl diuresis, this diuresis was promptly inhibited. Figure 1 is an example of such an experiment.

Only mersalyl diuresis, as shown by figure 2, was completely inhibited by BAL, while aminophylline diuresis, and sodium chloride diuresis in anesthetized rabbits were practically unaffected.

In anesthetized dogs, as in the rabbits, an intravenous injection of 5 to 10 mgm. per kgm. of mersalyl produced first a reduction in urine flow, followed by the

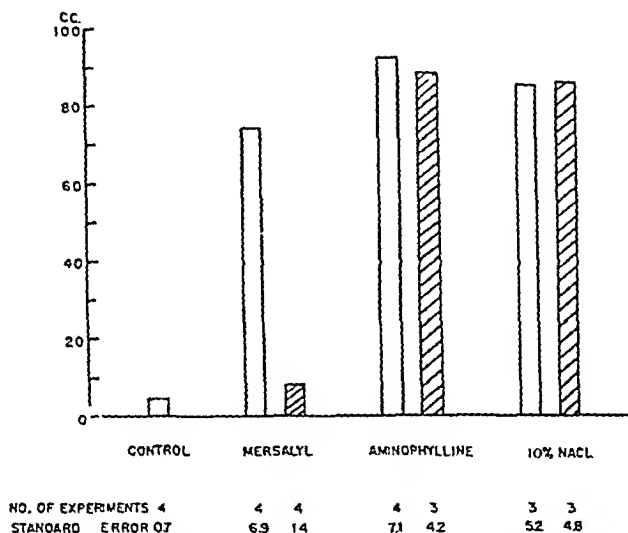


FIG. 2. THE EFFECT OF BAL ON MERSALYL, AMINOPHYLLINE, AND SODIUM CHLORIDE DIURESIS IN ANESTHETIZED RABBITS

Each column represents total volume of urine excreted in 90 minutes and is the average of the number of experiments stated below it. One rabbit was used for each individual experiment. White column—diuretic given alone intravenously (except in control experiments). Cross-hatched column—BAL in a dose of 10 mgm. per kgm. given intramuscularly 1 minute before the diuretic was given. The scale represents cc. of urine. Control—no diuretic and no BAL was given. Mersalyl—10 mgm. per kgm. intravenously. Aminophylline—15 mgm. per kgm. intravenously. 10% NaCl—5 cc. per kgm. intravenously.

characteristic diuresis. BAL in a dose of 1 to 2 mgm. per kgm. given either just before or after the appearance of mersalyl diuresis completely inhibited the diuretic activity of mersalyl.

To obtain quantitative information, experiments were performed on unanesthetized rabbits. They received 10 mgm. per kgm. of mersalyl intravenously and two minutes later a BAL solution was injected by the same route. The total 5 hour excretion of urine and chloride ion was determined. It can be seen from figure 3 that 2 mgm. of BAL (0.016 millimols) completely counteracted the diuretic effect of 10 mgm. of mersalyl (0.02 millimols), while 1 mgm. of BAL (0.008 millimols) was insufficient to suppress completely the diuresis pro-

duced by 10 mgm. (0.02 millimols) of mersalyl. Data obtained in the anesthetized dog (table 1) also show that approximately one SH-equivalent of BAL is required to counteract the diuretic action of one mole of mersalyl.

2. *The influence of monothiois on the diuretic action of mersalyl.* Cysteine hydrochloride and glutathione were tested in a manner similar to BAL in unanesthetized rabbits. Cysteine hydrochloride in doses up to 50 times those of

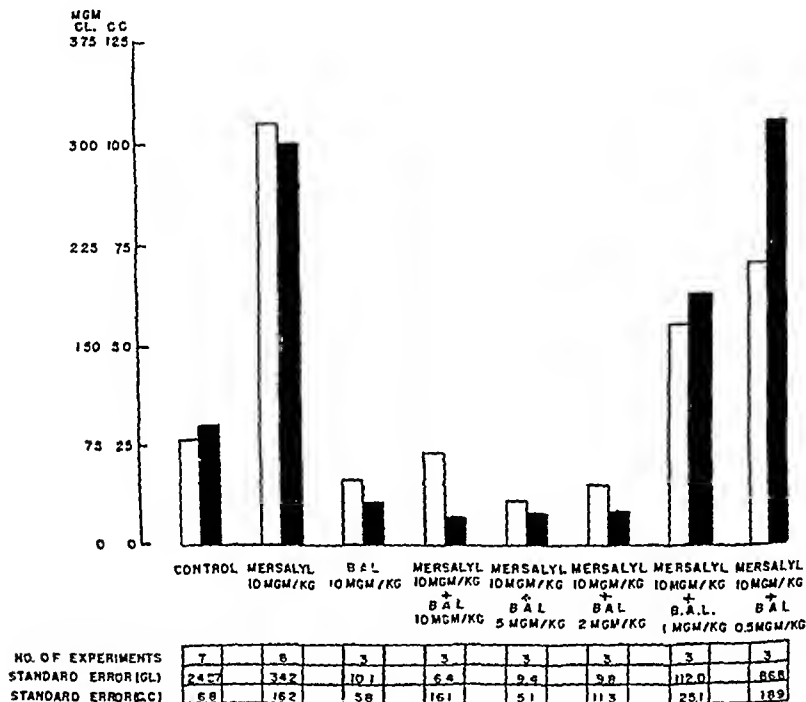


FIG. 3. THE EFFECT OF VARIOUS DOSES OF BAL UPON MERSALYL INDUCED DIURESIS IN UNANESTHETIZED RABBITS

The columns represent the average result of the number of experiments stated below them. Two animals were used for each individual experiment. White column—total urine excretion in 5 hours. Black column—total chloride excretion in 5 hours. The scale represents mgm. chloride and cc. of urine volume respectively.

BAL did not inhibit the diuretic action of mersalyl (figure 4). A dose of glutathione (2 experiments) about twenty times that of BAL was likewise unable to interfere with mersalyl diuresis.

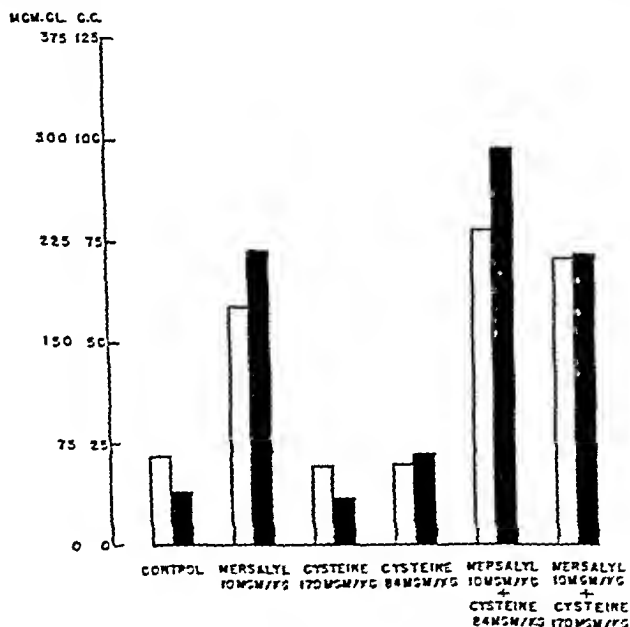
In the anesthetized dog cysteine and glutathione did not decrease the diuretic effect of mersalyl. In some experiments in dogs the observation was made that cysteine hydrochloride actually augmented the urine flow (see figure 5).

3. *Selective action of monothiois against cardiac toxicity of mersalyl.* Previous

observations (1, 2) have shown that cysteine, glutathione, and BAL could counteract the cardiotoxic action of mersalyl. To study the effect of these

TABLE 1
The Effect of BAL on Mersalyl Diuresis in the Anesthetized Dog

EXPERIMENT NO.	MERSALYL GIVEN		AMOUNT OF BAL NEEDED TO COUNTERACT MERSALYL DIURESIS	
	mgm.	millimols	mgm.	millimols
1	80	0.16	10	0.08
3	75	0.15	10	0.08
7	110	0.21	15	0.12
8	165	0.32	25	0.24
14	86	0.17	10	0.08



NO. OF EXPERIMENTS
STANDARD ERROR(CL)
STANDARD ERROR(CCL)

3	4	3	2	5	3
206	43.9	5.4	327	38.0	24.2
32	3.4	52	20	12.8	2.9

FIG. 4 THE EFFECT OF VARIOUS DOSES OF CYSTEINE HYDROCHLORIDE UPON MERSALYL INDUCED DIURESIS IN UNANESTHETIZED RABBITS

The columns represent the average result of the number of experiments stated below them. Two animals were used for each individual experiment. White column—total urine excretion in 5 hours. Black column—total chloride excretion in 5 hours. The scale represents mgm. chloride and cc. of urine volume respectively.

sulphydryl compounds on the cardiac and renal action of mersalyl simultaneously, blood pressure, electrocardiogram, and urine flow were recorded in anesthetized dogs (8 experiments). Mersalyl was given intravenously by a constant infusion

duced by 10 mgm. (0.02 millimols) of mersalyl. Data obtained in the anesthetized dog (table 1) also show that approximately one SH-equivalent of BAL is required to counteract the diuretic action of one mole of mersalyl.

2. *The influence of monothiods on the diuretic action of mersalyl.* Cysteine hydrochloride and glutathione were tested in a manner similar to BAL in unanesthetized rabbits. Cysteine hydrochloride in doses up to 50 times those of

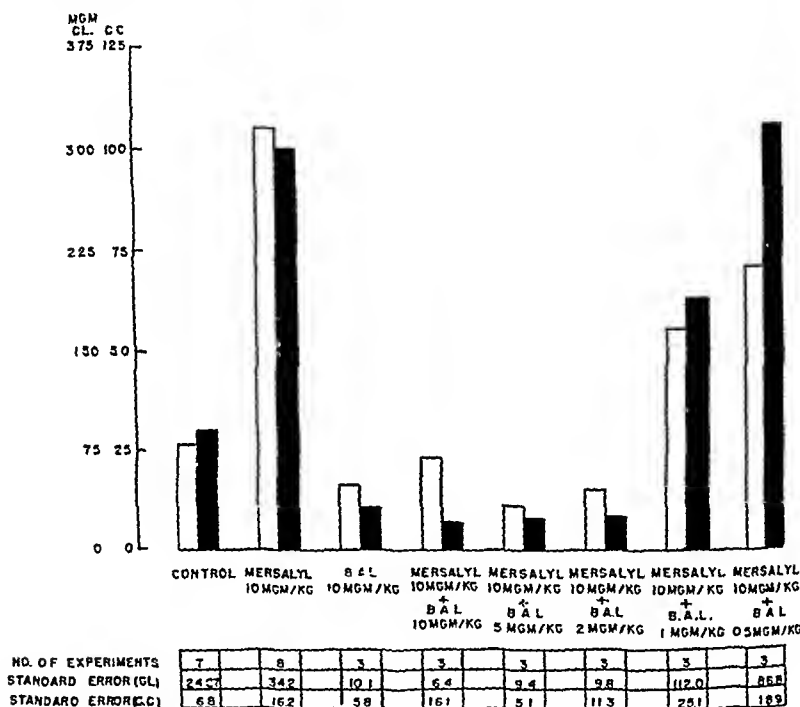


FIG. 3. THE EFFECT OF VARIOUS DOSES OF BAL UPON MERSALYL INDUCED DIURESIS IN UNANESTHETIZED RABBITS

The columns represent the average result of the number of experiments stated below them. Two animals were used for each individual experiment. White column—total urine excretion in 5 hours. Black column—total chloride excretion in 5 hours. The scale represents mgm. chloride and cc. of urine volume respectively.

BAL did not inhibit the diuretic action of mersalyl (figure 4). A dose of glutathione (2 experiments) about twenty times that of BAL was likewise unable to interfere with mersalyl diuresis.

In the anesthetized dog cysteine and glutathione did not decrease the diuretic effect of mersalyl. In some experiments in dogs the observation was made that cysteine hydrochloride actually augmented the urine flow (see figure 5).

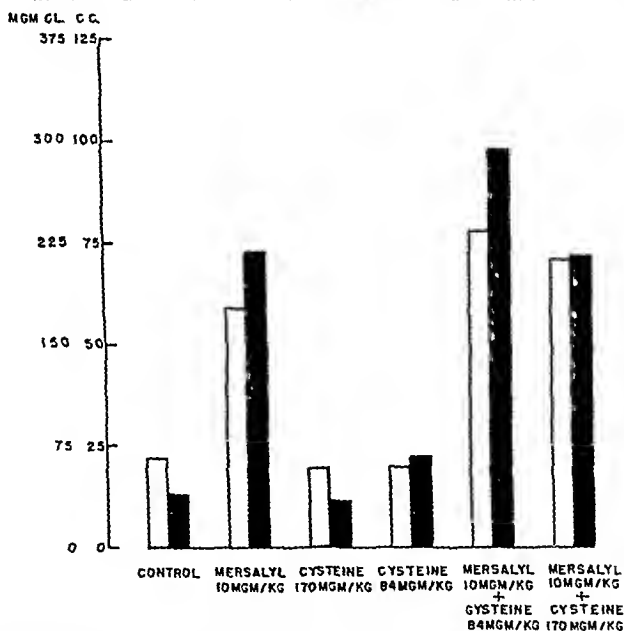
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	mgm	millimols	mgm	millimols
1	80	0 16	10	0 08
3	75	0 15	10	0 08
7	110	0 21	15	0 12
8	165	0 32	25	0 24
14	86	0 17	10	0 08



NO OF EXPERIMENTS
STANDARD ERROR(GL)
STANDARD ERROR(CC)

3	4	3	2	5	3
206	43.8	6.4	32.7	36.0	35.5
3.2	5.4	6.2	8.0	12.8	8.8

FIG 4 THE EFFECT OF VARIOUS DOSES OF CYSTEINE HYDROCHLORIDE UPON MERSALYL INDUCED DIURESIS IN UNANESTHETIZED RABBITS

The columns represent the average result of the number of experiments stated below them. Two animals were used for each individual experiment. White column—total urine excretion in 5 hours. Black column—total chloride excretion in 5 hours. The scale represents mgm chloride and cc of urine volume respectively.

sulfhydryl compounds on the cardiac and renal action of mersalyl simultaneously, blood pressure, electrocardiogram, and urine flow were recorded in anesthetized dogs (8 experiments). Mersalyl was given intravenously by a constant infusion

or by single injections. When severe cardiotoxic manifestations appeared the sulfhydryl compound was given intravenously.

Experiments conducted with BAL conclusively showed that this dithiol in doses counteracting the cardiac effect, also prevented or abolished the diuretic effect of mersalyl. Figure 5 shows the results obtained with cysteine hydrochloride. In his experiment mersalyl was infused into a dog at a rate of 1.0 mgm. per kgm. per minute. After about 20 minutes the diuresis began to diminish and after 20 mgm. of mersalyl per kgm. had been infused, cardiac irregularities appeared. The administration of 100 mgm. of cysteine hydrochloride

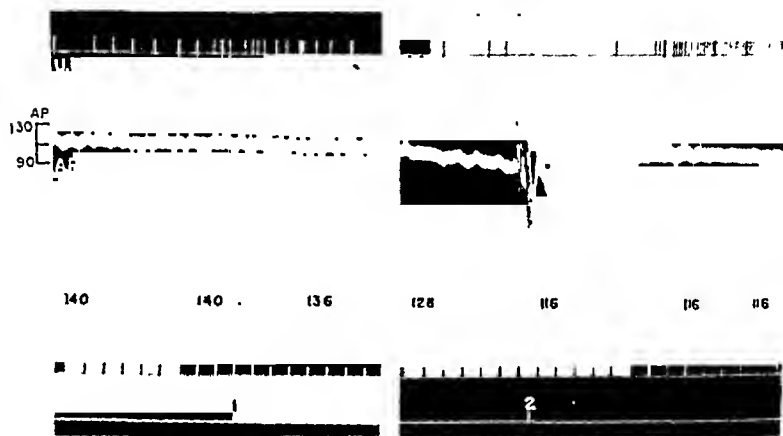


FIG. 5. THE EFFECT OF CYSTEINE HYDROCHLORIDE ON THE CARDIAC TOXICITY AND DIURETIC ACTION OF MERSALYL IN THE DOG

Female dog, 13.0 kgm. Anesthesia, dial-urethane, 0.7 cc. per kgm. intraperitoneally. U.F.—urine flow recorded with a drop recorder. A.P.—arterial pressure recorded with a mercury manometer from the left common carotid artery. Scale in mm. Hg. Numbers—heart rate per minute. Time in minutes. At 1, infusion of mersalyl started at a rate of 1.0 mgm. per kgm. per min. At 2, 100 mgm. cysteine hydrochloride injected intravenously. Time interval between first and second part of figure is 12 minutes.

promptly inhibited the cardiotoxic effects and the urine flow increased above that caused by mersalyl alone. Glutathione was like cysteine in that it counteracted the cardiac effects but did not abolish the diuretic effect of mersalyl.

It was shown previously (2) that a constant intravenous infusion of mersalyl given at a rate of 1.5 mgm. per kgm. per minute into anesthetized dogs resulted in severe cardiac arrhythmia after the administration of 23.2 mgm. per kgm. (8 experiments). The extent of protection afforded by cysteine against this action can be assessed from the following observations. If cysteine and mersalyl were administered simultaneously in a molar ratio of 1:1 (3 experiments) the irregularity dose became larger than 250 mgm. per kgm. An administration of mersalyl and cysteine in a molar ratio of 1:0.5 (3 experiments) resulted in an irregularity dose of 78 mgm. Similar data were obtained by Lehman (3) with the sulfhydryl compound thioglycollic acid.

4. *The initial antidiuretic action of mersalyl.* The intravenous injection of mersalyl resulted in a transient decrease in urine flow followed a few minutes later by the characteristic diuresis. This initial antidiuretic effect of mersalyl was observed both in rabbits and dogs and could be seen with single injections (see figure 1 and figure 6) as well as with constant infusion. With the latter method of administration and using rates of 1.0 to 1.5 mgm per kgm. per minute of mersalyl one may observe the antidiuretic effect of mersalyl only.

This antidiuretic effect promptly disappeared and a diuresis ensued when cysteine hydrochloride or glutathione were given intravenously. When mersalyl

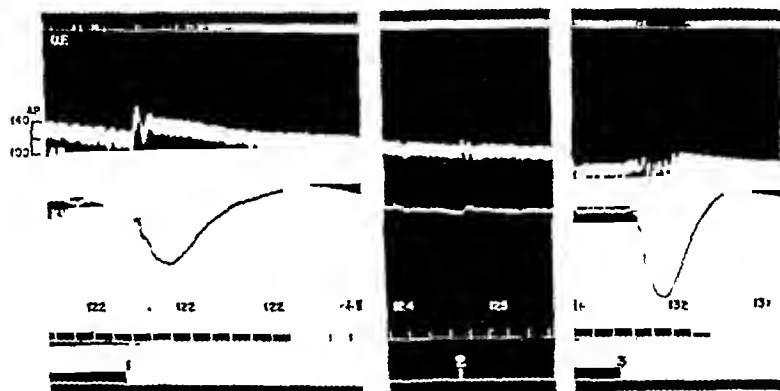


FIG. 6 THE EFFECT OF MERSALYL, AND A MIXTURE OF MERSALYL AND CYSTEINE HYDROCHLORIDE ON URINE FLOW, BLOOD PRESSURE, AND KIDNEY VOLUME IN THE ANESTHETIZED DOG

Male dog, 8.3 kgm. Anesthesia, 0.7 cc per kgm. dial urethane intraperitoneally. Tracings from above downwards: U F—urine flow recorded by a drop recorder, A P—arterial pressure, K V—kidney volume, time in minutes, Numbers—heart rate per minute. At 1, 83 mgm per kgm of mersalyl given intravenously. At 2, a mixture containing 83 mgm mersalyl and 40 mgm cysteine hydrochloride given intravenously. At 3, 83 mgm of mersalyl given intravenously. Time interval between first and second part of tracing, 6 minutes, between second and third part of tracing, 24 minutes.

and cysteine hydrochloride were administered simultaneously in a molar ratio of 1:1, this antidiuretic effect was not observed either with single injections (see figure 6) or with constant infusions.

It has been previously shown that the intravenous administration of mersalyl reduces kidney volume (6). We have confirmed this observation and have found that the initial decrease in urine flow was concomitant with the reduction in kidney volume (figure 6). Furthermore, giving cysteine hydrochloride or glutathione simultaneously with mersalyl, completely prevented the reduction in kidney volume (figure 6). Cysteine hydrochloride alone given intravenously in a dose of 2 to 10 mgm per kgm usually produced a slight increase in kidney volume. BAL given after the injection of mersalyl also abolished the kidney volume changes. An intravenous injection of BAL alone in a dose of 1 to 5 mgm per kgm reduced kidney volume slightly.

Since kidney volume changes are difficult to interpret, kidney blood flow was determined by means of a bubble-flowmeter (5 experiments). Some of the results obtained are illustrated in figure 7. Mersalyl given intravenously produced a marked reduction in kidney blood flow lasting 5 to 10 minutes; given intraarterially it was about 5 times as effective in reducing renal blood flow. Cysteine given simultaneously with mersalyl prevented this reduction in kidney blood flow (figure 7) just as it prevented the reduction in kidney volume; but without influencing the diuretic action of mersalyl. BAL in a dose of 1 to 2

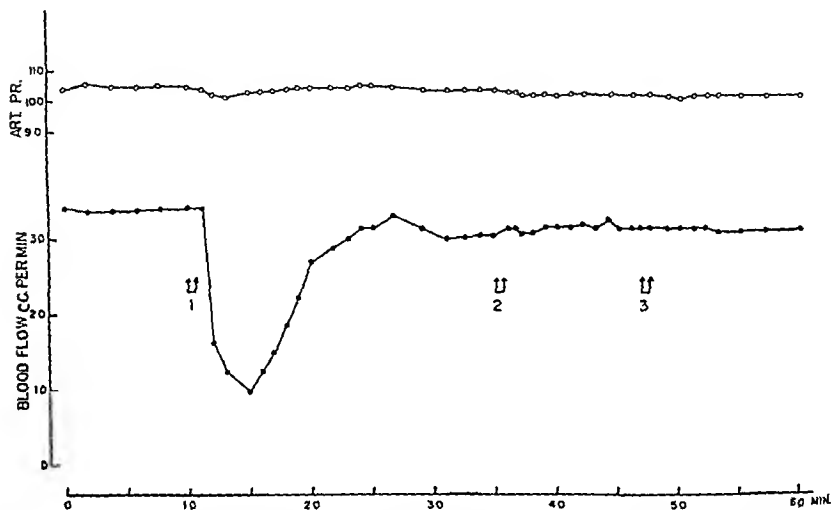


FIG. 7. ARTERIAL PRESSURE AND BLOOD FLOW THROUGH THE LEFT RENAL ARTERY IN THE ANESTHETIZED DOG

Female dog, 9.7 kgm. Anesthesia, dial-urethane, 0.7 cc. per kgm. intraperitoneally. Upper tracing—mean arterial pressure recorded from the left common carotid artery; scale on left in mm. of Hg. Lower tracing—blood flow through left renal artery; scale on left in cc. per min. At 1, 50 mgm. of mersalyl given intravenously. At 2, a mixture of 50 mgm. mersalyl and 25 mgm. cysteine hydrochloride given intravenously. At 3, 25 mgm. cysteine hydrochloride given intravenously. Time in 5 minute intervals.

mgm. per kgm. also counteracted the reduction in kidney blood flow produced by mersalyl but the diuretic action was also inhibited. Cysteine hydrochloride alone, in doses of 1 to 5 mgm. per kgm., did not produce any appreciable change in kidney blood flow, while BAL in a dose of 1 to 5 mgm. per kgm. given intravenously or intraarterially consistently caused a reduction.

Blood flow through the hind leg of the dog (4 experiments) was not appreciably influenced by mersalyl in doses ranging from 5 to 10 mgm. per kgm. given intravenously or intraarterially.

DISCUSSION. The diuretic action of mersalyl is counteracted by BAL and is abolished by the administration of half a mole of BAL for every mole of mersalyl. This suggests that the possible mechanism of action of the mercurial diuretics is

the inactivation of one or more SH containing enzyme systems responsible directly or indirectly for reabsorption of salt and water from the lumen of the kidney tubules.

Cysteine and glutathione do not interfere with the diuretic action of mersalyl in dosages up to 50 or 20 times respectively those of BAL. As in other biological systems (7), the monothiols are unable to reactivate the biochemical system with which the mercurial reacts in the nephron.

It has been previously shown (1, 2, 3) that mersalyl produces severe cardiotoxic effects which can be prevented or abolished by the dithiol BAL as well as by the monothiols cysteine, glutathione, or thioglycolic acid. Our experiments prove that contrary to the dithiol BAL the monothiols cysteine and glutathione in doses fully active against the cardiac effects, do not decrease urine flow in the normal animal, nor do they counteract the diuretic effect of mersalyl. Handy and LaForge (8) have shown that thioglycolic acid does not inhibit the diuresis produced by mercurhydrin. It appears possible therefore to utilize the selective action of monothiols like cysteine to prevent or abolish toxic cardiac effects without interfering with the desired diuretic action. This does not imply that all organic mercurials can be rendered non-toxic by either chemically combining them, or administering them together with sulfhydryl compounds. Cohen (9) has shown that alkyl mercuric thioglycolates can produce severe toxic central nervous system effects. Hence every single compound of this type requires careful investigation.

The characteristic diuresis caused by the intravenous administration of mersalyl is preceded by a transient reduction in urine flow. This has been correlated with a reduction in kidney volume and a decrease in kidney blood flow. The reduction in kidney blood flow is probably due to a local action of mersalyl on the kidney vessels, since intraarterial injections of mersalyl are more effective than intravenous injections. Blood flow through the hind leg of the dog is not appreciably changed by doses of mersalyl which produce striking effects on kidney blood flow. Hence different vascular areas appear to have a striking difference in sensitivity to the vasoconstrictor action of mersalyl.

The transient antidiuretic effect and the concomitant reduction in renal volume and in renal blood flow can be completely prevented or abolished by the monothiols as well as by the dithiols, which suggests a biochemical mechanism of action different from that involved in the inhibition of mersalyl diuresis and possibly similar to that involved in the abolition of the cardiotoxic effect of mersalyl.

SUMMARY. The diuretic action of mersalyl upon intravenous administration in the rabbit and in the dog can be counteracted by the dithiol 2,3 dimercapto-propanol (BAL). About half a mole of BAL is required to prevent or abolish the diuretic effect of one mole of mersalyl.

The monothiols cysteine and glutathione do not reduce the diuretic action of mersalyl. They protect the heart against the toxic effects of mersalyl, or abolish toxic cardiac effects already established, without inhibiting the diuresis produced by this compound.

The initial, transient decrease in urine flow, following the intravenous injection

of mersalyl, has been correlated with a reduction in kidney volume and a decrease in renal blood flow. The antidiuretic effect and the vascular changes in the kidney produced by mersalyl can be counteracted by the monothiols cysteine and glutathione as well as by the dithiol 2,3 dimercaptopropanol.

The authors wish to thank Dr. Otto Krieger for his advice throughout this investigation, and Mr. Henry George for technical assistance in a number of the experiments.

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THE EFFECTS OF SINGLE DOSES OF 6-DIMETHYLAMINO-4-4-DIPHENYL-3-HEPTANONE (AMIDONE, METHADON, OR '10820') ON HUMAN SUBJECTS

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The report of Scott and Chen (1) that 6-dimethylamino-4-4-diphenyl-3-heptanone (hereafter referred to as amidone) is a potent analgesic substance for animals and humans immediately raised the question of the addiction liability of the drug. Before studies of the addiction liability of amidone could be initiated it was necessary to carry out some preliminary investigations on the effects of single doses of the drug on human subjects. The purpose of this paper is to present the results of these studies.

Scott and Chen (1) found that the drug was a potent analgesic substance for rats, dogs and humans. The intravenous toxicity in mice was about twice that of demerol, but it was several times as potent in inducing analgesia. Eddy (2) confirmed the analgesic action of the drug in mice and found that the administration of 5 mgm./kgm. of amidone produced elevations of the pain threshold, as determined by the application of graded thermal stimuli, comparable to those seen after the administration of 10 mgm./kgm. of morphine sulfate. Amidone was approximately 10 times as toxic as morphine when administered subcutaneously to mice. Scott and Chen (1) also noted that most of the effects of the drug in animals were quite similar to those produced by morphine. Wikler (3) has found striking similarity between the actions of amidone and of morphine in all levels of the nervous system of dogs and cats.

The hydrochloride of amidone was the salt used in all our experiments. The drug was administered subcutaneously, unless otherwise noted. Except for a group of 10 non-addicts used in pain threshold experiments, all of the subjects for the experiments described below were former morphine addicts who had been abstinent from morphine for at least six months prior to being used as subjects for the experiment.

ANALGESIA. 2.5- to 5.0-mgm. doses were administered subcutaneously to 11 non-addicts and 2.5- to 15.0-mgm. doses were administered to 31 former morphine addicts. Pain thresholds were determined by the thermal radiation method of Hardy, Wolff and Goodell (4) except that the thresholds were expressed in terms of wattage input into the lamp (5) instead of calories/cm.²/sec. delivered to the irradiated area. At least 2 thresholds agreeing to within plus or minus 5 per cent were obtained before administering the drug. After the drug had been given, the pain thresholds were redetermined at convenient intervals until four hours had elapsed from the time of the administration. The results were expressed as percentage change in pain threshold from pre-injection thresholds. Changes in the thresholds were plotted against time using a scale such that 1 inch

of mercuryl, has been correlated with a reduction in kidney volume and a decrease in renal blood flow. The antidiuretic effect and the vascular changes in the kidney produced by mercuryl can be counteracted by the monothiol cysteine and glutathione as well as by the dithiol 2,3 dimercaptopropanol.

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The report of Scott and Chen (1) that 6-dimethylamino-4,4-diphenyl-3-heptanone (hereafter referred to as amidone) is a potent analgesic substance for animals and humans immediately raised the question of the addiction liability of the drug. Before studies of the addiction liability of amidone could be initiated it was necessary to carry out some preliminary investigations on the effects of single doses of the drug on human subjects. The purpose of this paper is to present the results of these studies.

Scott and Chen (1) found that the drug was a potent analgesic substance for rats, dogs and humans. The intravenous toxicity in mice was about twice that of demerol, but it was several times as potent in inducing analgesia. Eddy (2) confirmed the analgesic action of the drug in mice and found that the administration of 5 mgm./kgm. of amidone produced elevations of the pain threshold, as determined by the application of graded thermal stimuli, comparable to those seen after the administration of 10 mgm./kgm. of morphine sulfate. Amidone was approximately 10 times as toxic as morphine when administered subcutaneously to mice. Scott and Chen (1) also noted that most of the effects of the drug in animals were quite similar to those produced by morphine. Wikler (3) has found striking similarity between the actions of amidone and of morphine in all levels of the nervous system of dogs and cats.

The hydrochloride of amidone was the salt used in all our experiments. The drug was administered subcutaneously, unless otherwise noted. Except for a group of 10 non-addicts used in pain threshold experiments, all of the subjects for the experiments described below were former morphine addicts who had been abstinent from morphine for at least six months prior to being used as subjects for the experiment.

ANALGESIA. 2.5- to 5.0-mgm. doses were administered subcutaneously to 11 non-addicts and 2.5- to 15.0-mgm. doses were administered to 31 former morphine addicts. Pain thresholds were determined by the thermal radiation method of Hardy, Wolff and Goodell (4) except that the thresholds were expressed in terms of wattage input into the lamp (5) instead of calories/cm.²/sec. delivered to the irradiated area. At least 2 thresholds agreeing to within plus or minus 5 per cent were obtained before administering the drug. After the drug had been given, the pain thresholds were redetermined at convenient intervals until four hours had elapsed from the time of the administration. The results were expressed as percentage change in pain threshold from pre-injection thresholds. Changes in the thresholds were plotted against time using a scale such that 1 inch

represented 22.5 per cent change on one axis and 2.083 hours on the other. Thus, one square inch of the graph represented 46.87 "per cent hours" (2.083×22.5). The area under the curve was measured with a planimeter and converted to "per cent hours" (5) by multiplying the area in square inches times 46.87. The results are shown in tables 1 and 2. Elevations in the pain threshold were obtained in all the non-addict subjects. Negative values, indicating a fall rather than an elevation of the pain threshold, were obtained in 3 of 62 trials on former

TABLE 1

Effects of amidone and morphine on the pain thresholds of non-addict subjects
Results expressed in per cent hours

DRUG	DOSE	NUMBER OF SUBJECTS	MALES	FEMALES	CHANGE IN PAIN THRESHOLD	
					Average	Range
Amidone	2.5	7	4	3	+41.0	+ 8.4 to +76.7
Amidone	5.0	4	4	0	+50.0	+ 6.5 to +91.2
Morphine	5.0	2	0	2	+55.4	+48.6 to +62.6
Morphine ..	10.0	8	7	1	+33.9	-20.5 to +84.1
Normal Saline	1 cc.	5	3	2	+ 0.5	0.0 to + 2.7

TABLE 2

*Effects of amidone and morphine on the pain thresholds of former morphine addicts**
Results expressed in per cent hours

DRUG	DOSE	NO. OF SUBJECTS	CHANGE IN PAIN THRESHOLD	
			Average	Range
Amidone.. . . .	2.5	3	+ 6.6	- 4.5 to + 25.1
Amidone	5.0	20†	+42.4	-15.2 to + 90.0
Amidone ..	10.0	5	+70.6	+55.1 to +109.2
Amidone ..	15.0	5	+62.8	+33.8 to + 90.0
Morphine ..	10	13	+37.6	- 1.1 to + 90.0
Morphine..	15	5	+34.4	+83 to + 66.9
Morphine.	20	22	+66.6	+18.3 to +107.6
Morphine	30	27	+71.1	+ 5.9 to +153.0
Normal Saline	1cc.	14	- 0.9	- 1.3 to + 1.3

* All subjects were males.

† Average includes 62 tests on 20 subjects

morphine addicts. Two of these negative values followed 2.5-mgm. doses and one followed a 5-mgm. dose. Rises in pain threshold were obtained in all instances in which more than 5 mgm. were administered to former morphine addicts. The extent and duration of the elevation of pain thresholds found after 5-mgm. doses of amidone were higher than those found after the administration of 10-15 mgm. of morphine sulfate to both former morphine addicts and the non-addicts. Injections of normal saline produced very little change in the pain thresholds over four-hour periods of observation.

EFFECTS ON TEMPERATURE, PULSE, RESPIRATION, AND BLOOD PRESSURE. Pulse and respiratory rates, systolic and diastolic blood pressure, and rectal temperatures were determined on 10 former morphine addicts after they had rested quietly in bed for one hour. The subjects were then given 5-30 mgm. of amidone and the observations were repeated every thirty minutes for four hours. All the subjects continued to rest quietly in bed, but were not allowed to sleep. No consistent effects followed doses of 10 mgm., or less, of amidone. The respiratory rate was 3-8 per minute slower after 15-mgm. doses. The effect on the respiratory rate appeared sixty minutes after administration of the drug and persisted throughout the remainder of the period of observation. Pulse rates were slowed 4-10 beats per minute after doses of 10-30 mgm. of amidone. Respiratory rates did not fall below 12 and pulse rates did not fall below 40 in any of the subjects. Systolic blood pressures were reduced by 10 mm. of mercury after 30-mgm. doses. Diastolic blood pressures were not consistently affected. Rectal temperatures usually fell 0.1° to 0.4°C . These effects were probably not due to bed rest since they did not occur after doses of 10 mgm. or less.

ELECTROCARDIOGRAM. Electrocardiograms (3 standard limb leads and lead IV-F) were obtained before, and one and one-half to three hours after administration of 10- to 30-mgm. doses of amidone to 10 former morphine addicts. The only consistent effect noted in the electrocardiogram was the development of sinus bradycardia. The cardiac rate never fell below 50 in this series. Prolongation of the PR interval was never observed. No electrocardiographic evidence of toxic effects on the cardiac muscle was noted.

EFFECTS ON BLOOD SUGAR. Venous blood was drawn without stasis from 3 post-addict subjects and 30-75 mgm. of amidone were administered. After two hours the 2 men who had received 30 mgm., on examination failed to exhibit signs of the narcosis desired and an additional 30-mgm. dose of amidone was administered. About four and one-half hours after the original dose all 3 men showed evidence of strong narcosis. Venous bloods were again drawn. Bloods were analyzed for sugar by the method of Benedict (6) on Somogyi filtrates (7). The blood sugar level was unchanged in 2 subjects and decreased 15 mgm. in the 3d subject. Arterial bloods drawn at the same time as the venous bloods showed no change in sugar in any of the 3 men. In another experiment the blood sugar was followed at hourly intervals up to six hours after the administration of 45 mgm. to a single subject. No significant change occurred in the blood sugar levels of this patient. Similar tests on 4 former morphine addicts showed no change in blood sugar after the administration of 75-175 mgm. of morphine sulfate.

NAUSEA AND VOMITING. Nausea occurred 3 times and vomiting once in 13 trials of 5-mgm. doses in non-addict subjects. Sensations of light-headedness and faintness associated with pallor occurred twice in these subjects. Nausea and vomiting did not occur in 65 trials of 5-mgm. doses in former morphine addicts, but occurred once in 17 trials of 10-mg. doses. Of 10 post-addicts who received 30-mgm. doses of amidone, one was nauseated for forty-eight hours and vomited three times during that period.

SEDATION. This effect never was evident in the non-addict subjects who received 5 mgm., or less, and never was evident in former morphine addicts who received less than 10-mgm. doses. Sedation was sometimes observed with doses of 15-20 mgm. to former morphine addicts. Following the administration of 30-75 mgm. of the compound, sedation was regularly observed in the post-addict subjects, and was manifested by diminished activity, drowsiness, and by sleep from which the patient could easily be aroused. The onset of sedation was slower than that seen after morphine and could usually not be detected until one and one-half hours after administration of the drug. It appeared to reach its height from six to eight hours after administration of large doses and persisted for as long as forty-eight hours.

EUPHORIA. There was no evidence of the development of euphoria in either non-addicts or post-addicts with doses of 5 mgm., or less. Most individuals reported that, subjectively, they experienced no pleasurable sensations. A few subjects stated that they noticed a pleasurable sense of muscular relaxation and of mental ease. When doses of 10 mgm., or more, were administered to former morphine addicts objective evidence of euphoria appeared. Doses of 30 mgm., or more, always produced euphoria. The men became more at ease in the experimental situation; frequently they became talkative and boastful. They recounted their experiences as drug addicts and their successes in obtaining morphine and outwitting narcotic agents. They discussed the effects of the new drug at great length with each other and with the attendants. Usually they compared the sensations produced by amidone to those produced by the opiate drugs. They felt that subjective sensations were very similar to those induced by morphine or other opiate drugs but were slower in onset and longer sustained. Most of the men complained that amidone lacked the peculiar quality which they termed "drive" and which they defined as the ability of an opiate drug to produce ambition to work, to engage in games, listen to music, etc. When it was pointed out to them that, after a dose of morphine, their activities decreased, they did less work, were less interested in games and music, and actually went to bed where they remained in a semisomnolent state, they were puzzled and stated that after the administration of morphine they at least felt they were more ambitious, but that after the administration of amidone they knew they were not. All the former morphine addicts agreed that they preferred morphine or other opiate drugs to amidone because of the slower development of euphoria after amidone, and because of the lack of "drive". They all stated, however, that if opiate drugs were not available they would prefer amidone to alcohol, barbiturates, marihuana, or demerol.

In all these experiments the subjects were aware that the drug they were receiving was not morphine but a new synthetic drug and, since this knowledge may have effected their reaction to the medication, it was felt desirable to administer the drug to other former addicts without their having information as to what drug they were receiving. Doses of 10-30 mgm. were accordingly administered to a group of 5 former addict patients who had been receiving doses of morphine and other opiates at regular intervals for two weeks in studies on pain

thresholds. All of these men felt that they had received a dose of an opiate drug. One man said "That was a good shot, it must have been dilaudid. I think I got about one-sixth of a grain." None of these subjects appeared to be able to differentiate the subjective effects of amidone from those of morphine; they made no complaints and did not ask for morphine in place of the drug they had received.

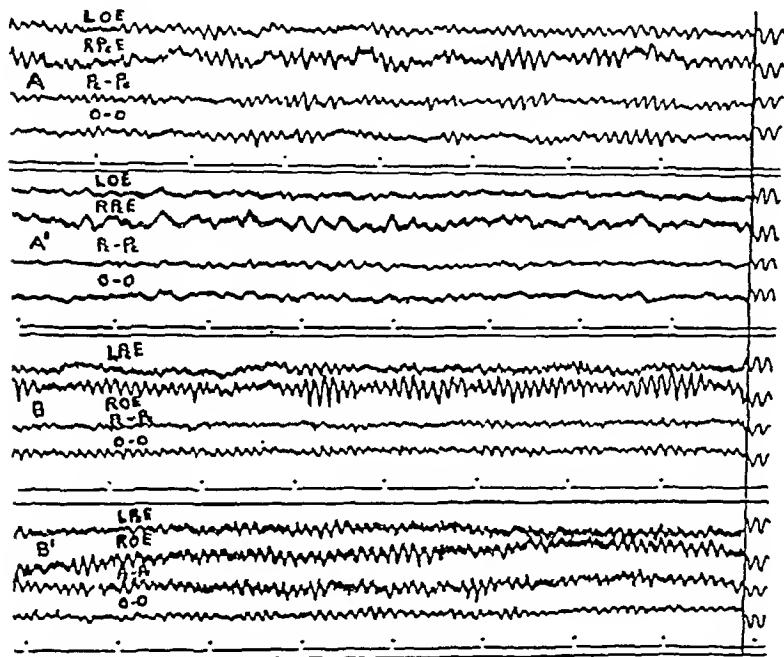


FIG. 1. EFFECTS OF SINGLE DOSES OF AMIDONE ON THE ELECTROENCEPHALOGRAM OF NON-TOLERANT SUBJECTS.

Leads: LOE, ROE—left and right occipital to ear (monopolar). LPcE, RPcE,—left and right precentral to ear (monopolar). PC-PC, left precentral to right precentral (bipolar), O-O, left occipital to right occipital (bipolar). Time in seconds. Calibrations 23.8 microvolts (shown at right). A, A', respectively control and one hundred minutes after subcutaneous injections of amidone, 30.0 mgm. in subject #723. Note marked slowing of record, with greatly increased, more or less continuous delta activity and practical disappearance of alpha frequencies. B; B', respectively control and ninety minutes after subcutaneous injection of amidone 30.0 mgm. in subject #722. Note absence of any significant change.

Doses of 10-30 mgm. were administered intravenously to 10 former morphine addicts. The men reported that the effects of the drug were similar to those produced by morphine or by heroin. They stated that they experienced sudden dizziness and peculiar abdominal sensations. They regarded these effects as highly pleasurable and stated that the drug was quite satisfactory. Flushing of the skin, comparable to that seen after intravenous injection of morphine, was noted in only 2 of the 10 patients. The comments of the men who received

intravenous doses were as follows: "That was great stuff. I wouldn't have believed it possible for a synthetic drug to be so like morphine. Can you get it outside? Will it be put under the narcotic law?"

EFFECTS ON THE ELECTROENCEPHALOGRAM. Electroencephalograms were made before and at various intervals up to two hours after subcutaneous injection of amidone in doses of 5.0- to 30.0 mgm. in 10 subjects, who reclined on a comfortable bed in an electrically-shielded, semi-soundproofed, air-cooled room in the presence of an observer who guarded against sleep, movement and other artifacts. Monopolar and bipolar tracings were obtained from the frontal, precentral, parietal and occipital regions. The reference electrode for the monopolar tracings was obtained from both ears which were connected together and grounded. It was found by experience that the frontal and precentral tracings behaved similarly, and this was also true for the parietal and occipital records. Hence, adequate records were obtained by simultaneous recording of one precentral monopolar, one occipital monopolar, precentral bipolar and occipital bipolar tracings. Four resistance-capacity coupled amplifiers were employed, the output from which activated four string galvanometers whose deflections were recorded optically on moving bromide paper.

In none of the subjects was any significant change noted with doses below 30 mgm. In one subject, however, 30 mgm. of amidone produced a marked shift of the frequency spectrum to the slow side (figure 1). Since such changes are identical with those seen in all subjects receiving repeated doses (8) it appears probable that larger single doses of amidone would produce changes in the electroencephalograms of all subjects similar to those noted in subject #723. No definite correlation could be made between the electroencephalographic changes noted in this subject and the comparative degree of sedation produced by the drug since this was difficult to measure.

DISCUSSION. Since 5 mgm. of amidone produces elevations in pain threshold comparable to those produced by morphine, it will likely be a useful analgesic drug. Our studies indicate that serious toxic reactions should not occur with dosages likely to be used in clinical practice. The side effects of the drug are somewhat similar to those produced by morphine but may occur less often. Kohlstaedt (9), Isbell et al (10), Kirchhof and David (11), and Troxil (12), have all reported that amidone produces good relief of pain in a large variety of clinical conditions.

Our data definitely show that the drug produces sensations which are highly pleasurable to former morphine addicts, particularly if administered intravenously. For this reason, it is certain that addicts will abuse amidone if it is freely available. Although euphoria has seldom been observed in clinical usage (9, 10, 11, 12), this potential action of the drug should be borne in mind and the dose limited in amount and frequency to the minimum required for pain relief.

SUMMARY

1. 5.0 mgm. subcutaneous doses of amidone elevated the pain threshold of non-addicts and former morphine addicts as much as 10-15 mgm. of morphine sulfate.

2. Respiratory rate, pulse rate, rectal temperature and systolic blood pressures were lowered by amidone.

3. 10-30 mgm. doses of amidone subcutaneously had no significant effect on the electrocardiogram.

4. 60-75 mgm. of amidone in divided doses had no significant effect on the blood sugar of former addicts.

5. 30-75 mgm. of amidone always produced sedation in former morphine addicts. The electroencephalographic pattern was sometimes shifted to the slow side after 30-mgm. doses.

6. 30 mgm., or more, of amidone subcutaneously regularly produced euphoria in former morphine addicts; 10-30 mgm. doses intravenously induced intense euphoria. Narcotic drug addicts would abuse amidone if it were freely available.

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THE BIOLOGICAL ASSAY OF HISTAMINE AND DIPHENHYDRAMINE HYDROCHLORIDE (BENADRYL-HYDROCHLORIDE¹)

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The recent discovery of potent practical antihistamine compounds has given a tremendous impetus to researches in experimental pharmacology and therapeutics in regard to the role of histamine in a number of physiological and pathological conditions.

The method of Barsoum and Gaddum (1) as modified by Code (2) and others has generally been adopted for the chemical isolation and the biological assay of histamine in blood and tissues. There is no method yet available in the literature for the quantitative determination of minute amounts of antihistamine compounds. Recently Gelvin and McGavack (3) applied the Brodie methyl orange technique for the estimation of Benadryl-HCl in body fluids. Staub (4), Rocha e Silva (5) and Loew and coworkers (6) have employed the isolated guinea pig's ileum for evaluating the relative potencies of various antihistamine substances by determining the amount of drug required to depress the histamine effect on the gut. We have developed a simple and rapid method using isolated guinea pig's ileum for the quantitative determination of small amounts of histamine and Benadryl-HCl.

Heretofore the biological assay of histamine was performed between an unknown and a standard by cross matching or by repeated graded responses (7). In the latter, the method is applicable if there is a linear relationship between log dose and effect over a given range. In the method to be described in the present paper the intestine is considered to be composed of a population of muscle fibers the response of which varies according to the frequency distribution of any other biological variate. The response to histamine following treatment with an antihistamine drug such as Benadryl-HCl is also graded in nature. Thus a linear relationship may be expected when the dose and response (in percentage) are expressed in terms of logarithm and probit units (8).

MATERIALS AND METHODS. A muscle bath² (fig. 1), calibrated in cc., was designed to meet the requirements of experimental conditions. For results to be reported in the following, the final volume of 4.3 cc. was used for histamine determination. That is, 0.3 cc. of histamine solution was added to 4.0 cc. Tyrode's solution in the bath, aerated with compressed air. By manipulation of the 3-way stopcocks, the bath was easily drained and refilled. In all experiments, the temperature of the bath was maintained at 38°C., and no spontaneous movements of the gut occurred (7) provided that certain precautions were taken in selecting the intestine.

It was absolutely necessary to use a clean quiescent ileum free from food material or gas in order to assure a uniform response of the gut to histamine and Benadryl-HCl. With a

¹ Benadryl-Hydrochloride—Parke, Davis and Company's Trademark for Diphenhydramine Hydrochloride.

² The bath was designed and constructed by Mr. V. A. Moore of these Laboratories.

little experience it was possible by careful inspection to select a satisfactory intestinal strip. Approximately 50 per cent of guinea pigs weighing around 300 grams and without food from 16 to 20 hours prior to sacrifice fulfilled these requirements. The 2 centimeter length of intestine just above the more muscular and enlarged portion of the ileocecal junction was used.

The strip was allowed to stand in the bath for 15 minutes to acclimate itself to the environment. It was then stimulated with an amount of histamine (0.3 or 0.4 micrograms of histamine diphosphate), which caused 40 to 80 per cent of the maximal contraction. The bath fluid was immediately changed with fresh Tyrode's solution after each contraction. This was followed by four more washings with fresh Tyrode's. The exposure of the gut to

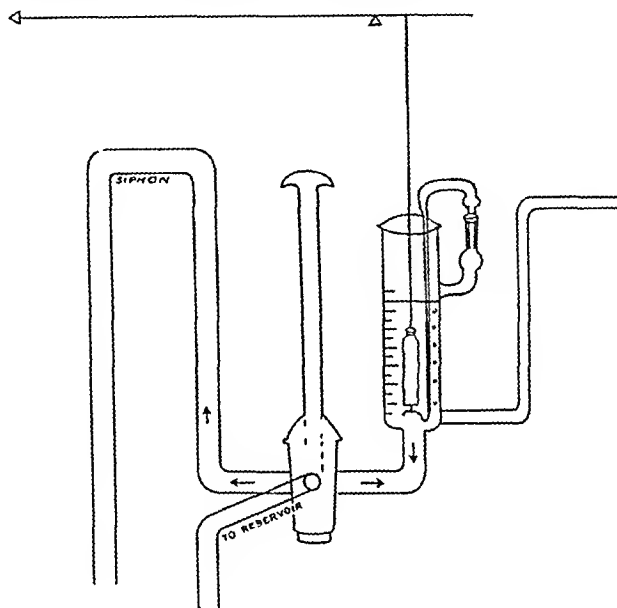


FIG 1

air during the short interval which elapsed between replacement of fluid did not cause spontaneous contractions or alter sensitivity to histamine. Repeated determinations were made at 3 minute intervals. After obtaining checks in triplicate determinations, the responses to graded doses of histamine diphosphate were carried out, beginning with 0.05 micrograms increasing in steps of 0.05 up to 0.3 and then with an increment of 0.1 microgram until a maximal contraction was obtained (fig 2). The concentration of histamine diphosphate causing a maximal response of the ileum in 56 experiments varied between 0.5 to 0.8 micrograms in 4.3 cc. As will be shown, the concentration of histamine producing a maximal response is immaterial in the calculation of response in percentages to graded doses of the drug provided it does not injure the tissue.

To ascertain the antihistamine effect of a drug, the uniformity in response of the gut to histamine was first determined. The lowest concentration usually 0.6 or 0.7 micrograms of histamine diphosphate in 4.3 cc. that would produce a maximal contraction of the intestine was used to determine the activity of Benadryl HCl. Three tenth cubic centimeter of Benadryl HCl solution was introduced into the bath to make a total of 4.0 cc. After one

minute a similar volume of histamine solution was added. Between successive determinations the gut was allowed to recover until it showed a normal response to histamine (fig. 2). This normal response to histamine determined each time just prior to Benadryl-HCl, and the response of the gut to the same concentration of histamine after addition of Benadryl-HCl were used to calculate their antagonistic effects.

In quantitating the biological responses, the contractions registered on the kymograph were measured in millimeters and were converted into percentages, based upon a maximal contraction. In histamine assay, the maximal contraction was taken to be 100 per cent; half of this was considered to be a 50 per cent response. In the evaluation of antihistamine activity, the difference in contractions to histamine before and after addition of Benadryl-HCl was used. Thus a 50 per cent difference in contractions represents a 50 per cent suppressive effect of Benadryl-HCl on histamine activity.

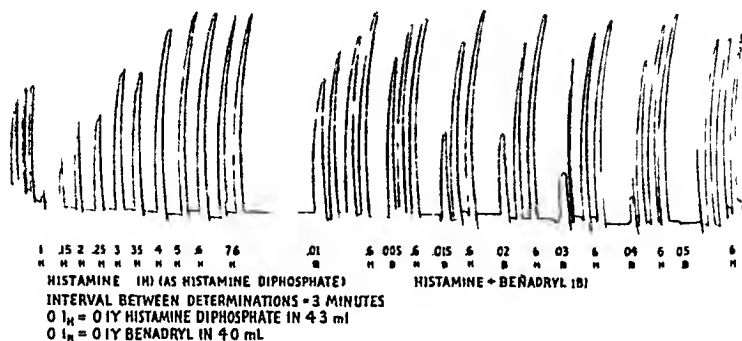


Fig 2 B = Benadryl, 1 minute later followed by 0.6 γ histamine diphosphate. Contractions without markings between B and H were produced by 0.6 γ histamine diphosphate after the Benadryl treated intestine had been washed several times with Tyrode's solution. The lower contractions as compared with that marked by 0.6 H indicate that the gut had not recovered to give a normal response.

In determining the concentration of histamine or Benadryl-HCl in samples containing unknown quantities, two dilutions (one twice the dilution of the other) that would produce a response between 10 to 90 per cent were assayed alternately with standard solutions of the respective compound. Four to six dilutions of the standard covering the entire range of responses were used. The concentration of the unknown was then interpolated graphically from the regression line between probits and log concentrations. It may also be calculated numerically as shown by Schild (7) and by Bliss (9), since here a linear relationship exists over the entire range between dose and response.

The lowest concentrations of the two substances that may be determined by the procedure are 0.05 γ /cc of histamine and 0.01 γ /cc of Benadryl-HCl. With certain modifications of the experimental technique, $\frac{1}{2}$ of above quantities may be estimated.

RESULTS AND DISCUSSION The records in fig 2 were obtained with the same intestine indicating the contractions to increasing concentrations of histamine and to the same amount of histamine following various quantities of Benadryl-HCl. It was found that antihistamine effect of Benadryl-HCl was still present after washing the gut 5 times with fresh Tyrode's and a resting period of 3 minutes. Following the influence of Benadryl-HCl in a concentra-

tion of 0.001 to 0.01 micrograms per cc. for one minute, the gut usually did not respond in the same degree to histamine until after several series of stimulation and washing (6 to 9 minutes). A gut treated with a completely suppressive dose of Benadryl·HCl often would not give rise to a normal maximal contraction to histamine for as long as 30 minutes or more. During successive estimations of Benadryl·HCl for quantities that suppressed the contraction of the gut to histamine more than 85 per cent of the maximal, the responses to the two drugs were very erratic. It would be of interest to know whether this is due to a slow releasing of Benadryl·HCl from the gut or due to an injury of the muscle tissue by the drug.

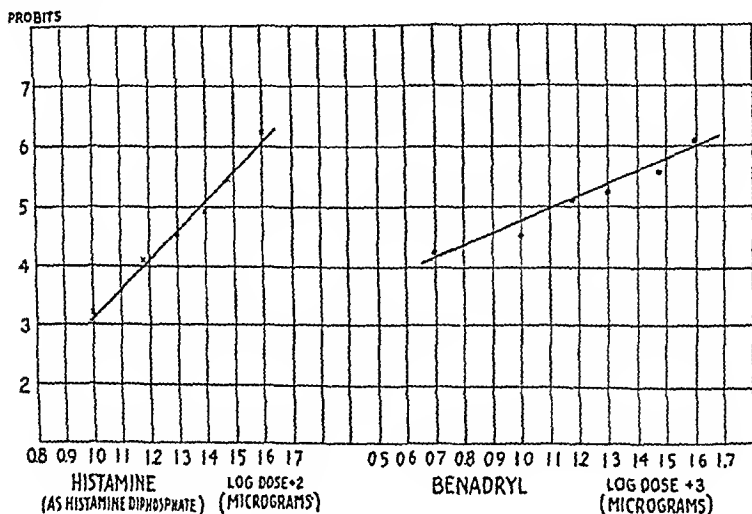


FIG 3.

The relationships between dose and response in figure 2 are shown in figure 3 in terms of probits and log doses. The graphs clearly indicate a linear relationship. Graphical estimation of the regression coefficient (the slope), standard deviation (the reciprocal of the slope) and the concentration of histamine or Benadryl·HCl will provide values sufficiently accurate for the determination of these substances in biological fluids. The equation for the regression line is: $y = \bar{y} + b_{yx}(X - \bar{X})$, where y and X are experimental values for response in probits and concentration in logarithm, b_{yx} the slope for regression of y on X , \bar{y} and \bar{X} the mean values of Y and X —in the present case equal to 5 (Probit) and Logarithm of the 50 per cent effective concentration of histamine or Benadryl·HCl respectively.

The results of 27 experiments on histamine alone and 29 experiments on histamine with Benadryl·HCl are presented as histograms in figure 4. The frequencies of slopes or the 50 per cent effective concentrations were plotted

against the range of variations. The values were obtained graphically from the regression line of each experiment, as shown in figure 3. With such a small number of determinations it is not possible to identify statistically the nature of the frequency-distribution of the variates. The mean slope and the mean 50 per cent effective concentration and their standard errors are respectively: 4.286 ± 0.161 and 2.750 ± 0.024 $\gamma/\text{cc. (Log)}$ for histamine diphosphate and 2.577 ± 0.187 and 3.660 ± 0.035 $\gamma/\text{cc. (Log)}$ for histamine with Benadryl-HCl.

The heterogeneity of the above series of regression coefficients was tested by an analysis of covariance with the procedure given in Goulden's text (10). The

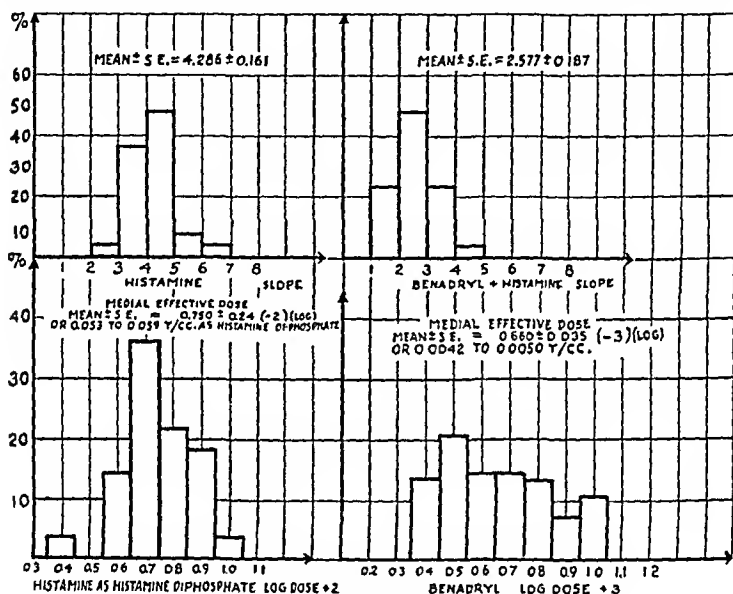


FIG. 4

results of 10 experiments, the regression coefficients of which as shown graphically cover the whole range of those in the entire series, were selected for statistical analysis. The deviations from the means were calculated by subtracting $5(\bar{y})$ from the experimental value of y in probits and subtracting the 50 per cent effective concentration \bar{X} , obtained graphically, from the corresponding value X .

Five or six determinations were available for calculating each regression coefficient by the method of least squares. The "F" values in Tables I and II indicate that the differences in regression coefficients are highly significant, most likely due to the differences in the intrinsic property of the muscle tissue of the ilia in response to histamine. In other words, the differences cannot be due to experimental errors. Therefore the assay of an unknown sample of histamine or Benadryl-HCl must be conducted by comparing with a standard on the same ileum.

It will be of theoretical interest and of practical importance to evaluate the various antihistamine agents by comparing the dose-response characteristics of their antagonistic effect toward histamine. The ratio of their 50 per cent effective concentrations will be a measure of the relative antihistamine potencies since the medial value (50 per cent) may be used for comparison irrespective of the nature of their dose-response relationship. A statistical difference in the regression lines or slope will indicate the similarity or difference in their mode of action toward histamine.

As shown by their medial effective concentration, the action of histamine may be antagonized by 1/10 equivalents of Benadryl-HCl indicating that the antihistamine effect of the latter is not due to a simple union between it and histamine.

TABLE I

The test of the heterogeneity of regression coefficients for histamine (10 experiments)

	df	$\Sigma (y')^2$ (ADJUSTED y)	VARIANCE	"F"	1%
Total	45	3.082			
Within each experiment.	36	1.015	0.028	8.19	2.91
			Error		
Difference	9	2.067	0.230		
			Regression coefficients		

TABLE II

The test of the heterogeneity of regression coefficients for Benadryl-HCl (10 experiments)

	df	$\Sigma (y')^2$ (ADJUSTED y)	VARIANCE	"F"	1%
Total	43	5.5751			
Within each experiment..	34	1.7235	0.051	8.39	2.97
			Error		
Difference	9	3.8516	0.428		
			Regression coefficients		

This supports the conclusions of previous workers (6, 11). The significant difference in regression coefficients to histamine without and with Benadryl-HCl (Table III) points to some other explanation (12). Benadryl-HCl may exert its action on certain enzyme systems upon which the effect of histamine is manifested.

To discuss the physiology of muscular contraction is beyond the purpose of the present paper. However, the dose-response characteristics of the ileum to histamine appear to be in favor of the "all or none" theory of muscular contraction (13). Since such data may be formulated as well in a linear relationship in terms of dose and logits as for a logistic curve, the quantitative response of smooth muscle to histamine may also be interpreted in some other manner (14).

In the determination of the antihistamine effect of Benadryl-HCl it was

observed that for an ileum very sensitive to histamine, i.e. giving rise to a greater response to a given amount of histamine than the average, a greater quantity of Benadryl·HCl was required to produce an average anti-histamine effect. This is shown by the correlation data in figure 5 where the medial effective concen-

TABLE III

The test of significance of the mean regression coefficients for histamine without and with Benadryl·HCl

TREATMENT	NUMBER OF EXPERIMENTS	df	MEAN REGRESSION COEFFICIENTS	SUM OF SQUARES
Histamine	27	26	4.236	18.9540
Histamine + Benadryl·HCl	29	28	2.577	29.4118
		Sum = 54	d = 1.709	Sum = 48.3658

$S = 0.946$, $S_d = 0.2526$, $t = 6.77$ (significant).

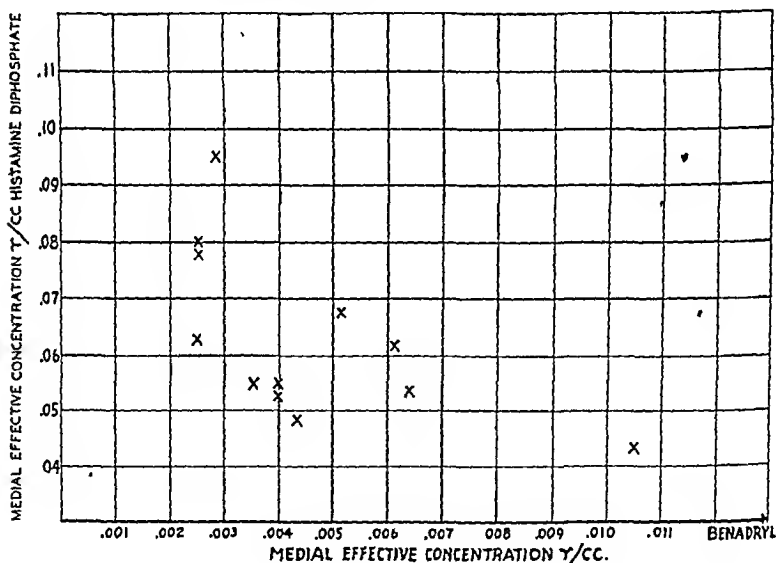


Fig. 5

tration of histamine was plotted against the medial antihistamine concentration of Benadryl·HCl for the same intestine.

In conclusion it may be stated that in the biological assay of histamine and the antihistamine effect of Benadryl·HCl the concentrations of these substances may be calculated either graphically or numerically from the regression lines when the percentage responses of the ileum to the drugs are expressed in terms of

probits and log doses. The regression coefficients and the medial suppressive concentrations of the different antihistamine agents may be utilized to compare their relative potencies and the mode of antagonism. The same principles may be applied to other biological assays and to studies of the mechanism involved in drug antagonism.

SUMMARY

A simple and rapid method of biological assay of histamine and Benadryl-HCl in vitro using isolated guinea pigs' ileum is presented. The linear relationship between dose and effect in terms of probits and log dose is utilized to calculate the unknown concentrations of these substances.

The mean concentration of histamine producing 50 per cent of the maximal contraction of an ileum was calculated from data of 27 experiments. The medial antihistamine concentration of Benadryl-HCl was determined in 29 experiments.

The heterogeneity of the regression coefficients of dose-response lines for histamine and for Benadryl-HCl was tested by the analysis of co-variance. The differences in regression coefficients were found to be highly significant.

Suggestions are given for applying the same principles to other types of biological assays and to study the mechanism involved in drug antagonism.

Acknowledgment. The authors are indebted to Dr. A. C. Bratton, Jr., for helpful suggestions during the course of this investigation.

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THE DETERMINATION AND THE URINARY EXCRETION OF 6-DIMETHYLAMINO-4,4-DIPHENYL-3-HEPTANONE HYDROCHLORIDE (AMIDONE)¹

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The new analgesic compound 6-dimethylamino-4,4-diphenyl-3-heptanone hydrochloride (Amidone) is at present studied in detail both pharmacologically and clinically and thereby has shown very promising results (1, 2, 3). However, these investigations did not cover the metabolism of this new drug since no simple method was available for its estimation in urine or body fluids. The only attempt to determine amidone in urine was made by Scott and Chen (1), but the method was not very sensitive and therefore not practical. In the present work a simple procedure for the estimation of amidone has been developed which lends itself to routine analysis.

The principle of the determination is based on the observation that many organic bases form with certain acidic compounds salt like addition products which are soluble in benzene or similar organic solvents and can thus be separated. If the organic acid is strongly colored, it may be measured colorimetrically and thus permit an estimation of the base. Most useful as acidic components are a number of indicator dyes. Thus Lehman and Aitken (4) used bromthymol blue for the determination of demerol, and Scott and Chen applied this method to amidone. Recently Brodie, Udenfriend and Dill (5) described the use of methyl orange in the estimation of cinchonine.

A series of preliminary experiments indicated that amidone forms benzene soluble addition compounds with several acidic dyes (Table I). In addition, the following dyes were tested at pH 7.5, 8.2 and 9.7, but did not react: eosine, fluorescein, phenolphthalein, phenol red, and thymolphthalein.

It is apparent that amidone reacts with all halogenated sulfonphthalein dyes but not with non-halogenated sulfonphthalein or phthalein dyes. Bromeresol purple was chosen for further studies because it has the greatest light extinction at the absorption peak, exceeding that of bromthymol blue by over 30 per cent. Furthermore, it has the advantage that the reaction with amidone proceeds at an acid pH which facilitates the required extraction by avoiding excessive emulsification.

It has been found that contrary to the procedure used by Lehman and Aitken (i.e.) in the determination of demerol it is not necessary to shake out amidone with benzene before reacting with the dye. In our experiments, we obtained very good results by adding the dye and the benzene directly to the properly buffered solution of the drug. The benzene will dissolve the addition compound

¹ Presented before the Division of Biological Chemistry of the American Chemical Society, New York, September, 1947.

but not the dye alone, and thus the former can be separated easily from the aqueous residue.

The amidone complex is decomposed by shaking the benzene solution with a dilute solution of sodium hydroxide, and the intensity of the resulting color solution is determined colorimetrically.

This method has given excellent results with pure aqueous solutions of amidone. Within the investigated range from 0 to 100 micrograms, the results follow Beer's law and are identical with those obtained with a pure dye solution.²

This indicates that the reaction proceeds quantitatively in the proportion of two moles of amidone for each mole of bromeresol purple. This proportion was to be expected since bromeresol purple contains two phenolic groups with acidic properties.

TABLE I
Acidic dyes reacting with amidone

ACIDIC COMPONENT	MAXIMUM LIGHT ABSORPTION		ADDITION COMPOUND WITH AMIDONE	
	λ in $m\mu$	Relative value	Formed at pH	Soluble in benzene
Bromeresol Purple	580	100	5.4	+
Bromthymol blue	610	75	7.5	+
Bromeresol green	620	65	5.2	+
Bromphenol blue	580	53	3.5	+
Bromochlorphenol blue	580	18	4.0	+
Chlorphenol red			6.5	-
Methyl orange	515		5.0	+

It may be mentioned that on a stoichiometric basis methyl orange would be advantageous since it forms only monobasic salts. However, the light absorption of methyl orange is considerably less than that of bromeresol purple so that the sensitivity is actually increased only by about 40 per cent. In addition, there are more interferences, and we therefore prefer the bromeresol purple method.

It has already been reported by Lehman and Aitken (i.e.) that urine contains basic substances which form benzene soluble addition compounds with bromthymol blue thus causing a blank reading of varying magnitude. These authors also found that this urine blank increases on standing and that it may be prevented by the addition of mercuric chloride.

In preliminary experiments, it was found that the normal urine blank with bromeresol purple may vary considerably from person to person and in the same person from specimen to specimen. Since the amidone concentration in urine is always rather small, the blank error may falsify the results by as much as 30%. Thus it was important that the blank could be actually determined in each sam-

² The assay data of the bromeresol purple were furnished through the courtesy of Mr. K. C. Reyen of the National Aniline Division, Allied Chemical and Dye Corporation, New York, N. Y.

of 0.29 mg. per cent while in all other subjects peak values were found ranging from 1.5 to 5.8 mg. per cent.

The figures for the urinary excretion of amidone ranging from about 6 to 13 per cent are lower than those reported by Scott and Chen (l.e.). We believe that this discrepancy is due to the fact that these authors did not determine the urine blanks so that their results are too high. Since they administered smaller doses of the drug than were used in our study the blank error is proportionally greater and this very well may account for the observed discrepancy.

A number of urine samples from patients receiving amidone were boiled under reflux with hydrochloric acid for varying periods up to three hours. Since this treatment did not change the measurable concentration of the drug it may be concluded that amidone is not excreted in conjugated form. This is not surprising if one considers that amidone contains neither a phenolic hydroxyl nor a free amino group on which conjugation usually takes place.

SUMMARY

A method for the determination of amidone has been described.

The urinary excretion of amidone has been studied in 5 patients. It has been found that only a small part of the administered drug is excreted unchanged.

The amidone used in this study was kindly supplied by Mallinckrodt Chemical Works.

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TETRAETHYLAMMONIUM CHLORIDE $[(C_2H_5)_4NCl]$. ACUTE AND CHRONIC TOXICITY IN EXPERIMENTAL ANIMALS

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The earlier literature on the pharmacodynamic action of tetraethylammonium compounds was summarized by Burn and Dale (1), Hunt and Renshaw (2), and Hunt (3). More recently Acheson and Moe (4, 5) and Acheson and Pereira (6) have studied the blocking of autonomic ganglia of the mammalian heart and circulation by tetraethylammonium. Loewi (7) reported on its antagonism to heart depressants. A series of publications have appeared on the effectiveness of tetraethylammonium chloride in the treatment of peripheral vascular diseases and causalgic states (7-12) and on its toxic manifestations in hypertensive persons (13). Toxicologic studies of tetraethylammonium compounds in animals are scant. Hunt (3) states that in the case of tetraethylammonium hydroxide subcutaneously in mice, the fatal dose is 0.107 mg./gram and that the approximately fatal dose for the chloride derivative is 0.12 mg./gram (2).

The object of this report is to present the toxicity, tolerance and histopathologic findings on tetraethylammonium chloride¹ in experimental animals.

Tetraethylammonium chloride $(C_2H_5)_4NCl$ is a quaternary ammonium compound, crystalline in nature. It is an odorless, chemically stable, hygroscopic substance, readily soluble in water. Toxicity studies were done using a 40.6 per cent commercial aqueous stock solution suitably diluted with distilled water. The reaction of a 10 per cent solution in distilled water was slightly acid, with a pH of about 6.0-6.5.

ACUTE TOXICITY. The acute toxicity of tetraethylammonium chloride was determined in normal albino mice, albino rats and dogs (Table 1). Chronic toxicity studies were carried out parenterally in albino rats and in dogs. The Dragstedt (14) double integration method was used for calculating the results.

On toxic doses, irrespective of the route of administration, the albino mice and rats developed tremors, incoordination and flaccid prostration. Death occurred from respiratory failure within 10 to 30 minutes.

In dogs, parenteral administration of lethal doses produced severe incoordination, flaccid prostration, respiratory and cardiac depression, marked ptosis and edema of eyelids, mydriasis, ocular muscle paralysis (inversion of eyeballs), erythema of ocular, nasal and less so of buccal membranes, paralysis of accessory respiratory muscles of chest, and death from respiratory paralysis and circulatory collapse. In a lower dose range the ocular changes, ptosis, mydriasis and erythema, and respiratory depression were of brief duration (15 to 60 minutes). Hyperpyrexia of one to four degrees Fahrenheit developed on intravenous ad-

¹ Available under the name of Etamon Chloride, P. D. & Co.

ministration. In a well tolerated dose range, 2.5 to 7.5 mg./kg. intravenously and 15 to 25 mg./kg. intramuscularly, the ocular changes were mild or only occasionally present.

CHRONIC TOXICITY. Albino rats in groups of 10 animals each were injected intramuscularly twice daily for 40 doses in a period of 22 days. Normal control rats received physiologic saline solution injections. Weight gains of individual animals were recorded at weekly intervals. Dosage to individual animals was adjusted at weekly intervals in proportion to the weight increase (Table 2).

TABLE 1
Acute toxicity of tetraethylammonium chloride

ANIMALS	ROUTE OF ADMINISTRATION	NUMBER OF ANIMALS	M.T.D.*	LD ₅₀
			mg./kg.	mg./kg.
Albino Mice . .	Intraperitoneal	536	37.5	65.0
" " .	Oral	235	250.0	900.0
Albino Rats	Intravenous	305	30.0	56.3
" " .	Intramuscular	80	75.0	110.0
" " .	Oral	155	750.0	2630.0
Dogs	Intramuscular	21	37.5	58.0
"	Intravenous	19	30.0	36.4

* Maximum tolerated dose (M.T.D.)—95 to 100 per cent of animals survive.

TABLE 2

Dosage and Gain in Weight in Rats Receiving (C₂H₅)₄NCl Intramuscularly Twice Daily for 40 Doses in 22 Days

	NUMBER OF ANIMALS				
	10	10	10	10	10
Mg./kg. twice daily.	15	25	50	130	Saline
Avg. initial wt. gms./rat	112.2	116.1	110.2	122.0	116.3
Avg. weight gms./rat at 3 weeks . .	179.4	170.3	174.0	—	185.0
Avg. gain in weight gms./rat	67.2	64.2	63.8	—	68.7
Mortality per cent	0.0	0.0	10.0	100.0	10.0

Animals receiving 15 to 25 mg./kg. twice daily remained free from reactions. With 50 mg./kg. twice daily the animals developed tremors and slight incoordination after each injection. The average gain in weight by animals was within the range of the gain by the control group of rats (Table 2). No cumulative toxic effects occurred. No death occurred at 30 or 50 mg./kg. in two divided daily doses. All animals receiving 130 mg./kg. twice daily died in 24 to 48 hours with symptoms of respiratory failure.

TOLERANCE OF TETRAETHYLAMMONIUM CHLORIDE IN DOGS, INTRAMUSCULARLY AND INTRAVENOUSLY. Normal adult dogs in groups of 2 to 6 were given tetra-

ethylammonium chloride twice daily intramuscularly and once daily intravenously five days each week, using a 20 per cent concentration for the first 2 to 3 weeks and a 10 per cent concentration during the remaining period of administration. Intramuscularly each site of injection was reinjected every 4th day. The animals were observed for local and systemic reactions. Blood counts, hemoglobin, total blood non-protein nitrogen, plasma protein determinations, and liver function tests were carried out at irregular intervals. Weight changes in animals were recorded each week.

Repeated intramuscular injections of 7.5 to 15.0 mg./kg. twice daily for 65 to 170 doses in 63 to 115 days caused no reactions, either systemic, gastrointestinal, or ocular. A dosage of 25 mg./kg. twice daily caused mild to moderate incoordination, ptosis of eyelids with or without slight edema, and hyperemia of the conjunctivae, the nares, and the buccal membranes. The erythema cleared up in about one hour after treatment. With increasing dosage (37.5 to 50.0 mg./kg. twice daily) the animals became severely incoordinate and developed severe respiratory depression after each administration. They recovered in 2 to 4 hours but a mild degree of residual muscular weakness and drowsiness persisted for about 3 to 4 hours.

Repeated intravenous administration of 2.5 to 10.0 mg./kg. daily was well tolerated except for transient ptosis of the eyelids, mydriasis, and erythema of conjunctivae in the higher dose range. In a still higher dose range, 15.0 to 30.0 mg./kg., the ocular changes became severe, transient paralysis of ocular muscles occurred, the vitreous humor infrequently appeared opalescent due to severe congestion of the ciliary body, choroid and retina. The reactions subsided rapidly and the animals appeared normal in 15 minutes to 2 hours. Intravenously to dogs tetraethylammonium chloride was about three to five times more toxic than intramuscularly.

HEMATOLOGY AND BIOCHEMISTRY. Dogs under tolerance studies were examined about every 2 to 3 weeks for changes in hematology, total blood non-protein nitrogen, total plasma protein, albumen, globulin fractions, bromsulphalein blood concentration and urine analysis using standard procedures. Blood samples were taken 24 hours following drug administration. No significant changes occurred in blood counts, hemoglobin, total blood non-protein nitrogen and total plasma protein values. The albumen and globulin values varied considerably from one test to another and from one animal to another without significant trend. The differential blood counts revealed no shift in cell morphology (Table 3). The efficiency of the liver for excreting dye was not diminished, the serum concentration of bromsulphalein at 30 minutes after administration remaining below 2.0 per cent. The urine remained free of albumen and sugar.

LOCAL TISSUE REACTION. A series of dogs and rabbits were injected with tetraethylammonium chloride intramuscularly, subcutaneously and intravenously to determine the local tissue reaction to 1 to 20 per cent aqueous solution.

Injections subcutaneously and intramuscularly of 1.0 to 2.0 cc. of 10 per cent aqueous solution in single or repeated doses caused no local tissue injury. A 2.5

cc. dose caused moderate edema and swelling which left no residual tissue damage at the end of 6 to 8 days. A dose of 5 cc. invariably caused edema and swelling for 24 to 48 hour periods. Autopsy on the 6th and 8th days revealed tissue ischemia, sclerosis, induration and a small necrotic area at the injected sites. The 20 per cent (C_2H_5)₄NCl locally produced necrosis of tissues with abscess formation.

Intravenous administration of 1.0 to 10.0 per cent concentrations caused no local sclerosis or thrombosis of the veins.

HISTOPATHOLOGY. Death of animals following parenteral administration of lethal or sublethal doses of tetraethylammonium chloride occurred within a few minutes to two hours from respiratory failure and circulatory depression. The underlying pathology of the tissues was that of severe congestion, blood stasis and petechial hemorrhages in visceral organs, liver, lungs, kidneys, spleen,

TABLE 3

Hematology, Non-Protein Nitrogen, Plasma Protein, Albumen-Globulin Ratio, Initial and Final Averages

NO. DOGS	DOSE MG./KG. DAILY	ROUTE OF ADMINISTRATION	NUMBER DAYS ON TREATMENT	R.B.C. IN MILL. CMM.		W.B.C. IN THOUS. CMM.		HGB. % (15.6 GR. = 100%)		N.P.N. MGS. %		PLASMA PROTEIN PER CENT		ALBUMEN-GLOBULIN RATIO	
				Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
3	15.0	I.M.	115	6.3	6.1	18.4	15.9	103	101	41	38	5.5	5.0	1.42	1.18
3	30.0	"	45.66	6.2	6.1	14.5	25.3	99	94	36	33	5.0	5.1	1.50	1.13
6	50.0	"	64.115	7.1	6.9	18.2	12.7	102	108	32	30	5.0	5.0	1.71	1.37
2	75.0	"	22.26	6.7	6.7	9.6	14.9	112	106	26	30	5.9	5.6	1.52	1.84
1	100.0	"	34	6.0	5.0	13.1	11.1	86	86	37	28	5.7	5.9	1.39	1.55
2	2.5	I.V.	42	7.0	6.6	14.1	11.3	105	103	37	34	5.6	4.9	1.69	1.35
2	5.0	"	42	6.7	6.9	14.6	11.6	109	109	42	38	5.4	5.4	1.76	1.71
2	7.5	"	42	7.3	7.4	13.1	13.9	116	116	39	37	5.2	5.4	3.06	1.63
2	10.0	"	42	6.0	5.7	16.3	14.4	99	99	37	38	5.8	5.3	1.13	1.64

gastro-intestinal tract, urinary bladder and brain. In addition, the liver frequently showed acute edema and necrotic cellular changes around the central vein of the lobule. In the kidneys, the convoluted tubules showed cloudy swelling, the lining of the peripheral collecting tubules (Henle's loops) was irregularly fragmented, vacuolated and in places desquamated. The thyroid and parathyroid glands, adrenals, pancreas and lymph glands appeared normal. The ciliary body, iris, choroid plexis and subcutaneous tissues of conjunctivae were congested. The optic nerve and retina appeared normal. Repeated daily administration in tolerated doses had a tendency to cause mild interstitial edema of the liver and kidneys, a mild hydropic degeneration of capillary cells of glomeruli, and a congestion of ocular membranes. No progressive degenerative changes were present. In the well tolerated dose ranges, up to 25 mg./kg. twice daily parenterally, the histopathologic changes were lacking or when present were temporary (edema and congestion) and disappeared on withdrawal of drug.

Pre-existing pulmonary or kidney complications in animals tended to accentuate the toxicity of $(C_2H_5)_4NCl$.

SUMMARY

Tetraethylammonium chloride $[(C_2H_5)_4NCl]$, an autonomic ganglionic blocking agent, was non-toxic perorally ($LD_{50} = 900$ to 2636 mg./kg.) and relatively toxic parenterally (36.4 to 110 mg./kg.) in animals.

It was well tolerated by intramuscular or subcutaneous administration of 15 to 25 mg./kg. twice daily in rats and dogs. Intravenously, dogs were free of reactions on 2.5 to 5.0 mg./kg. twice daily, while a dosage of 7.5 to 10.0 mg./kg. caused mild ocular symptoms of mydriasis, ptosis of eyelids and hyperemia of ocular, nasal and buccal membranes. Administration of progressively greater amounts resulted in incoordination, hyperpyrexia (intravenously), spasticity, respiratory and circulatory depression. Death occurred from respiratory failure and circulatory collapse.

The underlying histopathologic changes were those of severe congestion, stasis and anoxia. Lethal and sublethal single or multiple doses caused edema and degenerative changes in the liver and to a lesser degree in the kidneys. In tolerated doses the tissues were free of degenerative changes.

A 10 per cent aqueous solution of $(C_2H_5)_4NCl$ parenterally produced little or no local tissue injury. A 20 per cent concentration had a tendency to cause tissue necrosis.

No manifest changes occurred in hematology, total blood non-protein nitrogen, bromsulphalein liver function test, total plasma protein, albumen and globulin ratios, and urinalysis of dogs on daily or twice daily administration of tolerated doses.

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THE COMPARATIVE PHARMACOLOGY OF THE N-ALKYLARTERENOLS

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Since the fundamental study of Barger and Dale (1) of the relationship between the chemical constitution and physiological action of the sympathomimetic amines, numerous investigations have been made of many structurally and pharmacologically related compounds. Although several studies (2-9) have been concerned with dl-(3,4-dihydroxyphenyl)-2-aminoethanol or "Arterenol" and many (10) with its N-methyl homolog or epinephrine, comparatively few have investigated any of the higher N-alkyl homologs. Dakin (2), Loewi and Meyer (3), and Biberfeld (4) performed a few experiments with N-ethylarterenol and Raymond-Hamet (11-14) extensively investigated its actions.¹ Konzett (15, 16) reinvestigated the N-ethyl compound and discovered the intense bronchodilator activity of the N-isopropyl compound, in comparison with the n-propyl, n-butyl, and isobutyl homologs. Lands, Nash, McCarthy and Dertinger (17) also investigated N-isopropylarterenol. We have compared these various N-alkylarterenols with the parent compounds, arterenol and dl-epinephrine, and with two new compounds, N-secondary-butylarterenol and N-tertiary-butylarterenol.

1. BLOOD PRESSURE EFFECTS IN DOGS.

Twenty-two dogs were anesthetized by the intraperitoneal injection of 330 mgm. of sodium barbital per kgm. (10 per cent solution) 90 minutes prior to operation. Sixteen of these (six vagotomized, six atropinized with 1 mgm. of atropine sulfate per kgm.) had the carotid artery cannulated and connected to the usual mercury manometer, with the blood

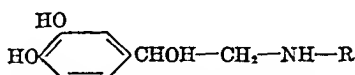
¹ Raymond-Hamet (11-14) called this compound "Ethylaminoethanolpyrocatechol" and Konzett (15, 16) "Ethyladrenalin." Both types of nomenclature have been employed in abstract journals. The name "Ethyladrenalin" is ambiguous and actually indicates the presence of both a methyl and an ethyl group on the nitrogen atom. This ambiguity could be avoided by using the "nor" prefix to indicate a lack of the methyl group and by calling the material "Ethylnoradrenalin" or "Ethylnorepinephrine". Unfortunately, however, both these names (42, 43) are used to refer to 1-(3,4-dihydroxy-phenyl)-2-amino-1-butanol, known also as "Butanefrin" and "Ethyl-norsuprarenin." Unquestionably the originator intended to call this material *alpha*-ethyl-norsuprarenin, to indicate chain substitution of the ethyl group and to differentiate from substitution on the nitrogen atom.

Similarly the indefinite term "Isopropyladrenalin" would indicate epinephrine with an additional isopropyl group on either the nitrogen atom or the "alpha" carbon atom. This material is also referred to in the literature as "Aludrine" and "Isuprel." In order to avoid this difficulty, and to prevent further confusion, we have called these compounds N-ethylarterenol, N-isopropylarterenol, etc., indicating the arterenol or norepinephrine molecule with the substitution of the appropriate alkyl group on the amino nitrogen atom. By this procedure, dl-epinephrine would be referred to as N-methylarterenol.

pressure changes recorded either on soot kymographs (seven) or on strip kymographs with ink-writers (nine). Six dogs (all atropinized) had the median circumflex branch of the femoral artery cannulated and the blood pressure changes recorded optically on photographic paper using either Hamilton (18) or Statham (19) manometers.

It is possible to give repeated injections of arterenol and of dl-epinephrine and to obtain virtually the same response in blood pressure effect (1). This is true only if the cardiovascular system is allowed to return to normal and if excessive doses (over 75 micrograms/kgm.) are avoided. The administration of graded doses of these agents yields typical response curves such as Hjort, de Beer and Randall (20) obtained with 1-epinephrine and 3,4-dihydroxyphenylethylmethylamine. The results from a typical dog have been plotted in graph

TABLE 1



COMPOUND	R	SALT	NUMBER	M. P. °C.
Arterenol	—H	HCl	—	153-4
dl-Epinephrine	—CH ₃	HCl	—	148-9
N-Ethylarterenol	—CH ₂ CH ₃	HCl	0-4, 1516	177-8
N-n-Propylarterenol	—CH ₂ CH ₂ CH ₃	Acetate	0-4, 1937	159-60
N-Isopropylarterenol	—CH(CH ₃) —CH ₂	HCl	0-4, 1024	171-2
N-sec-Butylarterenol	—CH(CH ₃) —CH ₂ CH ₃	Acetate	0-4, 1424	172-3
N-ter-Butylarterenol	—CH(CH ₃) —CH ₂ CH ₂ CH ₃	Acetate	0-4, 1505	185-6

1. Barger and Dale (1) found arterenol to be 1.25 to 1.5 (1.43) times as potent as dl-epinephrine. Most of our dogs gave pressor responses to arterenol and dl-epinephrine that were virtually equal (see figure 1). The dog used for the graph was more sensitive to arterenol and gave the typical results that have led to the discrepancy in the reported relative potency of these two agents (1, 4, 5). At the low end of the curve, the two agents are relatively equipotent, but as the dose is increased, arterenol becomes more and more active.

Our results with N-ethylarterenol are in close accord with those of Raymond-Hamet (14). Figure 1 illustrates typical results. Very small doses (1 to 2.5 micrograms/kgm.) usually give only a slight, transient fall in blood pressure. As the dose is increased, a transient rise in blood pressure appears, followed by a more prolonged fall. The major portion of the fall occurring with doses of

100 micrograms or more per kgm., lasts only a few minutes, but the blood pressure never completely returns to normal after these large doses. The n-propyl homolog produces a typical small rise, followed by a small reduction in blood pressure. It can be seen from graph 1 that it is both less effective as a vasopressor and vasodepressor agent than the ethyl compound. The isopropyl homolog does not produce much pressor response, but it is a very potent depressor agent. Typical examples of its action are given in figures 2 and 3. In the graph, only the depressor activity is included. The quantitative relationships between dose and the amount of fall in blood pressure are apparently dependent in part on the condition of the animal. In animals with high normal blood pressure, small doses (less than 2 micrograms/kgm.) are appreciably depressor

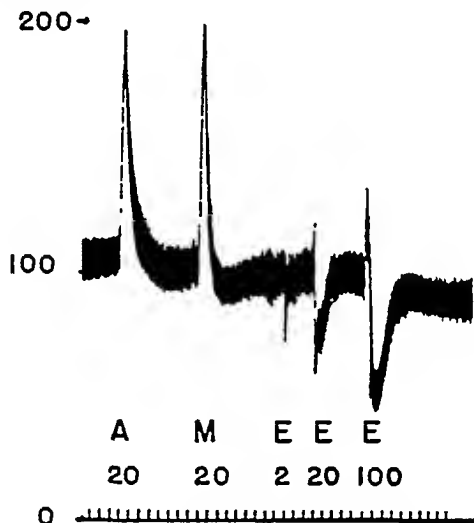


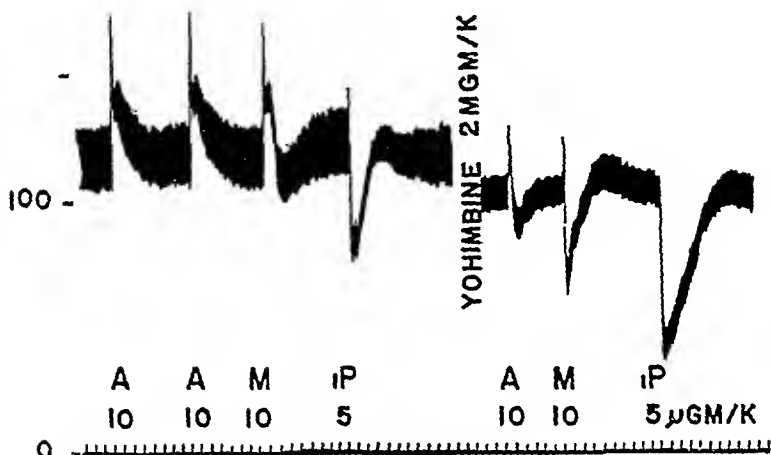
FIG. 1. Dog (♀; 8 ком.)

All doses given in micrograms/kgm. Arterenal hydrochloride injected intravenously at A, dl-Epinephrine hydrochloride at M, and N-Ethylarterenal hydrochloride at E. Pressure scale in mm. Hg.; time scale in minutes.

in effect (as much as 40 mm. Hg. mean pressure), and the result is quantitatively reproducible after the blood pressure has returned to normal. If the agent depresses blood pressure more than 60 mm. Hg., or if the animal is in poor condition, the return to normal is very slow and often incomplete. In animals with low blood pressure (less than 80 mm. Hg. mean pressure), considerably larger doses of agent are required to produce an equivalent depressor effect. In this respect the agent is similar to histamine, which in small doses produces reproducible falls in blood pressure in animals in good condition (22).

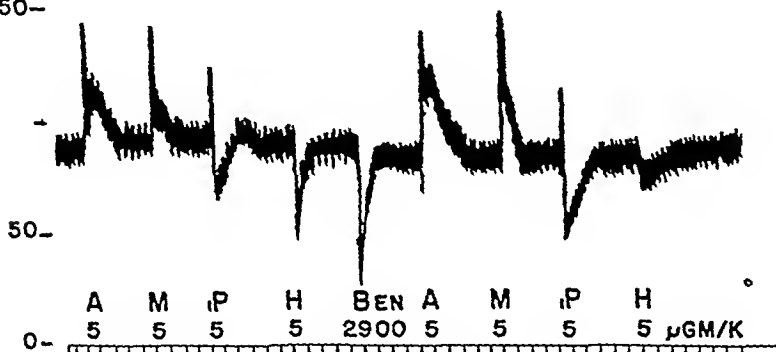
The secondary-butyl compound is not particularly potent, lying between N-ethyl- and N-isopropylarterenal in depressor activity (see Graph 1). It has little and sometimes no pressor activity, much like the n-propyl homolog.

200 -

FIG. 2. Dog (σ ; 12 kgm.)

Atropinized. All doses given in micrograms/kgm. unless otherwise indicated. Kymograph run continuously except for 6 minute period during which yohimbine was injected. Arterenol hydrochloride injected intravenously at A, di-Epinephrine hydrochloride at M, and N-Isopropylarterenol at iP. Pressure scale in mm Hg.; time scale in minutes.

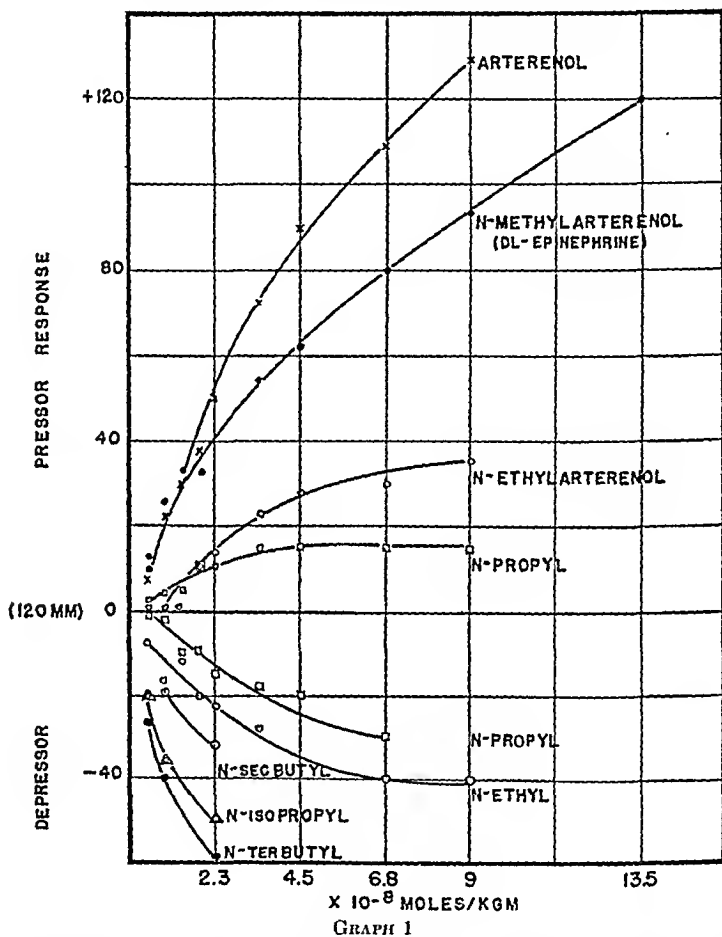
150-

FIG. 3. Dog (σ ; 10 kgm.)

Neither atropine nor vagotomy was employed. All doses given in micrograms/kgm. Arterenol hydrochloride injected intravenously at A, di-Epinephrine hydrochloride at M, N-Isopropylarterenol hydrochloride at iP, Histamine phosphate at H, and Benadryl hydrochloride at Ben. Pressure scale in mm Hg.; time scale in minutes.

However, the N-tertiary butylarterenol is the most potent depressor agent of the group, being 40 to 60 per cent more active than the isopropyl compound. It is interesting to note that the most potent depressor agents are those whose longest straight chain on the nitrogen atom is one with two carbon atoms (0-4),

1516; 0-4, 1024; and 0-4, 1505) while those with more than two (0-4, 1937; 0-4, 1424; and the n-butyl and isobutyl compounds) have considerably less



GRAPH 1

Dog (♀; 9 kgm.). 330 mgm. of Na Barbitol per kgm. Mean normal blood pressure = 120 mm. Hg. Data plotted from soot kymograph record of injections given 8 to 13 minutes apart. The procedure of Barger and Dale (1) of comparing agents on an equimolecular basis has been used (1×10^{-8} mole is equivalent, in micrograms per kilogram, to 2.06 arterenol HCl, 2.20 of dl-epinephrine, 2.34 of N-ethylarterenol, 2.71 of N-n-propyl, 2.48 of N-isopropyl, and 2.85 of the butyl compounds). The dl-epinephrine assayed 0.55 as potent as USP XI (l-) epinephrine in 3 dogs. This is in good agreement with Cushny (0.54) (21), Barger and Dale (0.65) (1), and Schultz (0.66) (5).

activity. On the other hand, the pressor activity apparently decreases linearly as the length of the alkyl group on the amino-nitrogen atom.

2. BLOOD PRESSURE EFFECTS IN CATS. Since much of the earlier blood pressure studies with some of these compounds was done with cats, we anesthetized

5 cats with 330 mgm. of sodium barbital per kgm., vagotomized, cannulated the carotid artery, and recorded blood pressure changes with a mercury manometer and soot kymograph. Qualitatively, the results are very similar to those obtained in the dog. Quantitatively, there are a few differences. Arterenol, in doses of 10 micrograms or more per kgm., is 30 to 50 per cent more active as a pressor agent than is dl-epinephrine in similar doses. However, 2 to 5 times as much ethyl, isopropyl, or tertiary butylarterenol are required to produce the same fall in pressure as was obtained in the dog. The only obvious conclusion is that the cat is more sensitive to vasopressor agents and comparatively less sensitive to vasodepressor agents (23).

3. BLOOD PRESSURE EFFECTS IN RABBITS. Six rabbits were anesthetized with 1.5 grams of urethane per kgm. given intraperitoneally and the carotid blood pressure was recorded with a mercury manometer and a soot kymograph. The rabbits gave pressor responses to arterenol and to dl-epinephrine that are about one-half that expected, at 5, 10, and 20 micrograms/kgm. as compared to the dog. The effects of N-ethyl, n-propyl, and isopropylarterenol are quantitatively identical with those in the dog, while the responses to the N-sec-butyl and ter-butylarterenol are considerably decreased and in most instances are less than those obtained with the isopropyl compound. All six of these rabbits gave only pressor responses to 50 micrograms of histamine phosphate per kgm. (22).

4. MECHANISM OF BLOOD PRESSURE ACTIONS. At the present time, it is fairly well accepted that the cardiovascular effects of epinephrine are due to increased cardiac output (increased force of contraction, increased stroke volume), cardiac acceleration, generalized arteriolar constriction, and to some type of capillary or other peripheral vasodilation (10, 24, 25, 26). Depending on dose, the result of administration of epinephrine may be a small fall in blood pressure (from a small dose producing transient vasodilation) or a rise in blood pressure followed by a small, prolonged fall (from an integrated combination of all effects). Arterenol has been differentiated from epinephrine in that the vasodilator effects are much less or are non-existent (7-9). Figure 2 is typical.

It is relatively easy to determine the presence of peripheral vasodilation by examination of the diastolic slope of the aortic pulse contours (29, 30), if adequate manometers are used and the periphery is unobstructed. In dogs so prepared, there is very little change in blood pressure following the administration of 0.5-1.0 microgram of dl-epinephrine per kgm., but usually the rate of diastolic run-off increases and often there is the disappearance of the typical standing waves. This effect is not observed with arterenol, but it is apparent with similar doses of N-ethylarterenol, and with as little as 0.1 microgram of N-isopropyl or of N-ter-butylarterenol per kilogram. As well as this diffuse peripheral vasodilation that occurs with small doses, large doses produce obvious vasodilation of skin vessels, as evidenced by the characteristic reddening of the skin. Burn (23) has adequately reviewed the evidence concerning sympathetic vasodilator fibers, and although such effectors do certainly exist and can have appreciable action, it is difficult to conceive that they have the ability to produce the

pronounced and prolonged falls in blood pressure that occur with the larger doses of N-isopropyl and N-ter-butylarterenol. It has been questioned that all the epinephrine reversal effect can be due to sympathetic vasodilator fibers (10), and it may well be that some direct action on smooth muscle of capillaries or arteriovenous capillary shunts may be involved.

4a. Effect of Adrenolytic Agents. Various drugs such as the ergot alkaloids (25), yohimbine (27), or 883F and 933F (28) antagonize the vasopressor action of epinephrine so that the vasodilator action is apparent. Arterenol presumably has so little vasodilator activity that no typical reversal occurs after these drugs (6-9). In figure 2, arterenol has diminished pressor activity after yohimbine, and a very slight depressor effect, but nothing to compare with the fall produced by epinephrine, although they both had almost identical actions on the cardiovascular system before the yohimbine administration. Following the administration of 1-3 mgm. of yohimbine hydrochloride or of 2-4 mgm. of 883F or of 933F per kilogram, the minor pressor effects of the various ethyl, propyl, and butyl homologs are diminished and in some cases abolished, while the depressor effects are in all cases intensified. Figure 2 illustrates the phenomenon using N-isopropylarterenol as an example.

4b. Cocaine Sensitization. It was found by Tainter (6) in cats and by Melville (8) in dogs that cocaine potentiates the pressor effect of arterenol even more than that of l-epinephrine. We had considerable difficulty obtaining results that could be reasonably discussed, since the blood pressure either fell after the cocaine administration (15 mgm./kgm. subcutaneously or 5 mgm./kgm. intravenously) or rose to new levels, so that comparisons with the standard doses could not be made. In 2 of 5 dogs that maintained approximately normal blood pressure, cocaine potentiated (40 to 50 per cent) the pressor response of all the drugs studied; the depressor response was potentiated slightly (10 to 15 per cent). These results are consistent with either concept of cocaine sensitization, i.e., that it acts by inhibiting oxidation of sympathicotropic agents or that it increases permeability so that more drug reaches the effector (31).

4c. Benadryl. Benadryl (32-34) has the ability to abolish the vasodepressor actions of histamine and anaphylactoid agents with similar effects. It is also known to potentiate the effects of epinephrine. If the vasodepressor effects of these agents are mediated through the same effector system as histamine, then Benadryl should antagonize them. Figure 3 illustrates typical results obtained before and after the administration of 2.9 mgm. of Benadryl hydrochloride per kgm. The pressor effects of arterenol and of dl-epinephrine are slightly potentiated, the depressor effects of N-isopropylarterenol are potentiated in amount and duration, and the effect of histamine is typically antagonized. Thus, these depressor agents cannot act through the same effector pathway, mediator substance, or muscle system as histamine.

4d. Atropine. Atropine often potentiates the effects of epinephrine, primarily by preventing the reflex slowing of the heart mediated by the vagus nerve. One would scarcely expect these N-alkylarterenols to produce vasodepressor effects through action on the parasympathetic or cholinergic systems. How-

ever, to rule out this possibility, we administered these agents in dogs before and after atropinization (1-2 mgm. of atropine sulfate per kgm., intravenously) and found neither measurable increase nor decrease in the depressor effect, indicating that these agents do not act through the effectors of the parasympathetic nervous system.

5. PERFUSED ISOLATED HEART. The optical records of the effects of these agents in atropinized dogs show increased heart rate and probably increased force of cardiac contraction. The hearts of 4 cats and of 5 rabbits were perfused with Ringer-Locke solution by a modified Langendorf procedure. The agents all produce cardioacceleration, increased cardiac output, and increased coronary outflow as indicated in table 2.

6. BRONCHODILATOR ACTION. We employed the method of Tainter (35), using perfused isolated guinea pig lungs constricted with 0.01 per cent histamine

TABLE 2

Effect of N-alkylarterenols on perfused heart and lung preparations

	RABBIT HEART PERFUSATE CONCENTRATION FOR 100% INCREASE			CAT HEART CONCENTRA- TION FOR 100% INCREASE		GUINEA PIG LUNG CON- CENTRATION TO ANTAGO- NIZE 100 µgm HISTAMINE
	Rate	Contraction	Coro- nary flow	Rate	Contraction	
Arterenol..	1000-2000	1000-2000	1000	200	500-1000	500-700
dl-Epinephrine....	1000-2000	1000-2000	1000	200	500-1000	100-120
N-Ethylarterenol..	500-1000	500-1000	250	100	250-500	30-50
N-n-Propylarterenol	1000-2000	1000-2000	1000	200	500-1000	300-500
N-Isopropylarterenol	300-500	300-500	200	20-30	75-100	10-30
N-sec-Butylarterenol.	250-500	500-1000	200	75-100	200-500	30-50
N-ter-Butylarterenol.	200-300	200-300	100	10-15	30-50	8-12

All concentrations are expressed in micrograms of free base per liter of oxygenated perfusion fluid.

(as diphosphate). All these agents are potent bronchodilators (see table 2). Tainter (35-37) found arterenol $\frac{1}{2}$ to $\frac{1}{4}$ as active as l-epinephrine in antagonizing bronchospasm produced by histamine and arecoline. Considering that we used dl-epinephrine instead of l-epinephrine, our comparative ratios for the ethyl and isopropyl compounds are approximately one half those Konzett found in dogs (15) and are in fair agreement with those of Lands (17).

7. ISOLATED SMOOTH MUSCLE SEGMENTS. The quantitative effects of these agents on typical smooth muscle segments suspended in oxygenated Ringer-Locke solution and recorded by the Magnus technique are given in table 3.

8. TOXICITY IN MICE. We administered the agents under consideration intraperitoneally and intravenously to white mice of 16-23 grams weight in groups of 10. The data are summarized in table 4. Regardless of route of administration, some animals died of pulmonary edema from arterenol and dl-epinephrine, while others died of unknown causes following considerable vaso-

constriction (eyes bulged) and convulsions. The higher homologs, that have less vasopressor activity, are considerably less toxic.

9. EFFECTS IN MAN. The obvious possible therapeutic usefulness of the higher homologs for the relief of bronchial asthma made it desirable to obtain information concerning their action in normal man. Dautrebande (39, 40)

TABLE 3
Effect of N-alkylarterenols on isolated tissue segments

	RABBIT JEJUNUM		GUINEA PIG ILEUM CON- TRACTED WITH 500 µgm./l. HISTAMINE	GUINEA PIG UTERUS	GUINEA PIG UTERUS CON- TRACTED WITH 100 µgm./l. HISTAMINE
	Complete inhibition of spontaneous contraction Micrograms /liter	Per cent inhibition by 100 µgm./liter	Per cent inhibition by 500 µgm./liter	Complete inhibition of spontaneous contraction Micrograms/ liter	Per cent inhibition by 200 µgm./liter
Arterenol.....	125	50	100	500	20
dl-Epinephrine.....	100	100	100	200	100
N-Ethylarterenol.....	200	25	90	200	30
N-n-Propylarterenol....	>1000	10	30	>1000	50
N-Isopropylarterenol....	100	100	50	200	100
N-sec-Butylarterenol....	>1000	10	10	1000	100
N-ter-Butylarterenol....	>1000	10	10	200	100

All concentrations are calculated in terms of free base.

TABLE 4
Lethal effects of N-alkylarterenols in white mice

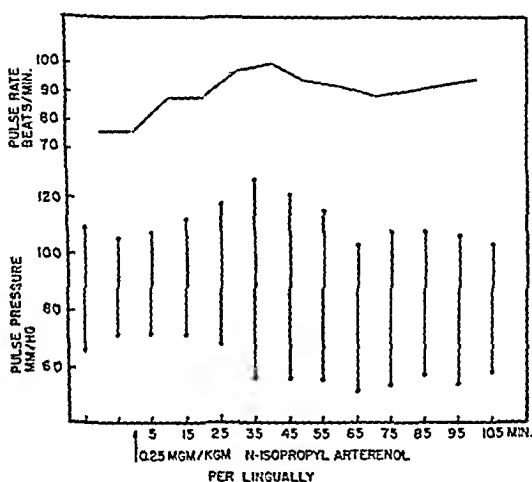
	LD 40-60 IP	KONZETT SC (16)	LANDS IP (17)	LD 40-60 IV	REPORT IV (41)
Arterenol.....	12-30	—	10	5	—
dl-Epinephrine.....	7-10	—	4	5	5.7
N-Ethylarterenol.....	10-15	6	27	8-12	—
N-n-Propylarterenol....	150-300	200+	—	—	—
N-Isopropylarterenol....	120-300	75	450	50-60	—
N-n-Butylarterenol.....	—	>200	—	—	—
N-Isobutylarterenol....	—	>200	—	—	—
N-sec-Butylarterenol....	180-300	—	480	>100	—
N-ter-Butylarterenol....	180-300	—	—	>100	—

All doses in micrograms/Gram body weight of mouse.

has indicated that N-isopropylarterenol can be administered as an aerosol. The report (38, 17) that this agent can be given orally or perlingually to develop its typical bronchodilator effect would indicate a more convenient mode of administration than is possible with epinephrine.

A series of 8 experiments on normal human adults, male and female, weighing 48 to 80 kgm., was performed. Systolic and diastolic blood pressure and pulse

rate were determined by the common sphygmomanometer cuff procedure in upright individuals. Experiments were started not less than $2\frac{1}{2}$ hours after a light meal, and repeated not oftener than once a week. In preliminary experiments, 0.25 mgm. of dl-epinephrine, N-ethylarterenol, N-isopropylarterenol, or N-tertiary-butylarterenol per kilogram, were administered as powdered drug under the tongue, after a control period of 30 to 45 minutes. This dose of epinephrine and of N-ethylarterenol had virtually no effect. The N-isopropylarterenol usually produced a slight rise in systolic blood pressure followed by a more prolonged fall in diastolic blood pressure, and customarily an increase in heart rate. A typical experiment is reproduced in graph 2. The individual seldom complained of untoward internal symptoms, although temporary feelings



GRAPH 2

Human female (50 kgm.). 12.5 mgm. of N-Isopropylarterenol hydrochloride placed under tongue at time indicated by arrow. Blood pressure determined sphygmomanometrically from left arm.

of warmth, flushing, and tingling of the face were common. This dose of the N-ter-butylarterenol produced precipitous falls in diastolic blood pressure which in turn produced weakness, prostration, nausea and vomiting. The individual complained bitterly of general malaise for some 2 hours after obvious symptoms had disappeared.

Oral administration of this dose of dl-epinephrine produced cramp-like pains in the stomach (apparently from localized vasoconstriction) but no measurable effects on blood pressure. The N-ethylarterenol had no effect from 2 to 4 hours following oral administration and then produced considerable lowering of diastolic blood pressure. Apparently there was localized vasoconstriction that limited its absorption. The N-isopropylarterenol had very similar effects whether given orally or perlingually. Effects quantitatively similar to 0.25

mgm. of N-isopropylarterenol per kgm. were obtained following 0.10 mgm. of N-ter-butylarterenol per kgm., given orally.

One asthmatic individual obtained relief from symptoms for a period of 48-72 hours following one perlingual administration of 0.25 mgm. of N-isopropylarterenol per kgm., while under comparable circumstances, relief for only 4-6 hours was obtained from 1:100 epinephrine aerosol. It was found that relief for 12-24 hours could be obtained from doses of 0.05-0.10 mgm. of N-isopropylarterenol per kgm., without noticeable changes in the cardiovascular system. These results are entirely in accord with the various bronchodilator experiments.

Discussion. The extreme potency of l-epinephrine and its probable rôle in the mediation of the sympathetic nerve impulse might seem to preclude the possibility that other agents could have even greater sympathomimetic action. It cannot be denied that dl-arterenol has greater excitatory action than dl-epinephrine, and in some instances even more than l-epinephrine, and consequently one of the isomers of arterenol must be much more active than l-epinephrine. This is no reason to conclude that arterenol, and not epinephrine, is the mediator substance, or to postulate that arterenol is Sympathin E, or excitatory sympathin (8, 9). In the first place, we have no knowledge of what fraction of injected epinephrine reaches excitatory effectors to produce a rise in blood pressure. Some of it is taken up by inhibitory effectors, and some of it is probably adsorbed by indifferent structures. Arterenol is simply available to the excitatory effectors in a higher proportion of the dose administered, since it has virtually no inhibitory action and may presumably not be taken up by inhibitory effectors, and, of course, less of it may be lost to other channels. As the size of the substituent on the nitrogen atom is increased from methyl to ethyl, the effect is a shift from a predominantly excitatory effect to one that is more nearly equal with respect to excitation and inhibition. With the isopropyl and ter-butyl alkyl groups on the nitrogen in place of the methyl, the agent is predominantly inhibitory and only a vestige of excitatory activity remains.

That some specific spatial limitations are involved in the effectors is absolutely necessary, since inhibitory effectors will not respond to Sympathin E, which presumably is epinephrine and some added substance, while excitatory effectors will not respond to Sympathin I, which is presumably the same epinephrine and some different added substance. Thus, the excitatory effectors will accept particularly those molecules in this group that have one or no carbon atoms on the amino nitrogen, and poorly those that have two, while agents with carbon chains of three or more carbon atoms are virtually ineffective in this regard. The inhibitory effectors will particularly accept those molecules in this group that have two-carbon-atom chains (ethyl, isopropyl, ter-butyl), and to a lesser degree those with more (n-propyl and butyl) or less (epinephrine) than two, and least those with none (arterenol).

That these differences cannot be due to some simple factor such as enhanced oxidation, increased excretion rate, or differential absorption, is well shown by the experiments with the various isolated organ structures which have a pre-

dominant sensitivity to one type of action or the other (usually inhibitory) and no ability to influence their environment, and yet the relative activity of these agents is still of approximately the same order.

SUMMARY

The effects of dl-(3,4-dihydroxyphenyl)-2-aminoethanol (Arterenol) and its nitrogen alkyl homologs, methyl (dl-epinephrine), ethyl, isopropyl, n-propyl, sec-butyl, and ter-butylarterenol, have been determined with the usual pharmacological test objects, including man.

These agents have both excitatory and inhibitory actions, with arterenol and dl-epinephrine having the greatest excitatory activity, the ethyl some, and the others little; while the isopropyl, ter-butyl, and ethyl have the greatest inhibitory activity, the methyl some, and the others little, with arterenol very little or none.

Acknowledgements. We are grateful to M. L. Tainter of the Winthrop Chemical Company, New York, for the Arterenol and dl-epinephrine; to M. L. Moore of Frederick Stearns and Company, Detroit, for the other N-alkylarterenols; to D. Bovet of the Institut Pasteur, Paris for the 883F (Prosypal) and 933F; and to G. Rieveschl, Jr., of Parke, Davis and Company, Detroit for the Benadryl.

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SUMMARY

The effects of dl-(3,4-dihydroxyphenyl)-2-aminoethanol (Arterenol) and its nitrogen alkyl homologs, methyl (dl-epinephrine), ethyl, isopropyl, n-propyl, sec-butyl, and ter-butylarterenol, have been determined with the usual pharmacological test objects, including man.

These agents have both excitatory and inhibitory actions, with arterenol and dl-epinephrine having the greatest excitatory activity, the ethyl some, and the others little; while the isopropyl, ter-butyl, and ethyl have the greatest inhibitory activity, the methyl some, and the others little, with arterenol very little or none.

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INFLUENCE OF BAL ON THE TOXICITY AND CHEMOTHERAPEUTIC ACTIVITY OF MAPHARSEN¹

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The effective detoxification of arsenicals by BAL first reported by Peters, Stocken and Thompson (1) is part of a more general phenomenon determined by compounds containing an SH group, known since 1923 by the work of Voegtlin and coworkers (2, 3). On the basis of Voegtlin's findings referring to such compounds, one would anticipate that not only the toxic effect, but also the therapeutic activity of the arsenical would be decreased by BAL. A certain indirect support is given to this expectation by observations made *in vitro* by Eagle (4), who found that trypanosomes and spirochetes made immotile by the arsenical regained their motility after the addition of BAL while the arsenical was removed from the parasite and passed into the supernatant fluid. On the other hand, it is in contrast to this expectation that the BAL-Mapharsen compound has been found parasitocidal by Peters and Stocken (5) and by Friedheim (6).

As yet, no direct information is available on the influence of BAL treatment on the chemotherapeutic activity of the arsenical. We studied the influence of BAL on the toxicity and chemotherapeutic effectiveness of Mapharsen from a quantitative viewpoint, mainly to determine the direction in which the therapeutic index is shifted by the combined treatment.

MATERIAL AND METHODS. Albino mice of varying weights (18 to 30 grams) and both sexes were used. Mapharsen (Parke, Davis & Co.) was injected in physiological solution and BAL in 0.5 cc. of peanut-oil. Injections of both of these materials were made subcutaneously on the back and always at different sites. In preliminary experiments it had been found that the drug was better tolerated, and the detoxification was more effective, if BAL was given in 2 subdivided doses at 2 hour intervals. Therefore, this treatment schedule was used throughout: the total dose of Mapharsen and half of the BAL dose were given simultaneously; the second half of the BAL dose was injected 2 hours later.

For infection, mice were injected intraperitoneally with 0.5 cc. of a suspension containing 10 *T. equiperdum* per microscope field (400 magnification). Untreated mice infected with this inoculum of parasites died within 3-4 days. Treatment was started 44-48 hours after infection, when all mice showed trypanosomes in the blood. Microscopic examination for parasites was done daily: surviving animals were kept in observation for a minimum of 30 days.

I. Detoxification experiments. (a) To have comparative data for evaluation of the experimental findings, it was necessary to determine accurately the toxic doses of Mapharsen and BAL under our experimental conditions. The following

¹ Part of this material has been presented at the 47th General Meeting of the Society of American Bacteriologists in Philadelphia, Pa., in May, 1947.

figures ensued: LD₅₀ for Mapharsen 42.5 mgm./kgm.; for BAL, given in 2 doses 2 hours apart, 2 x 125 mgm./kgm.; the MTD for BAL is 2 x 80 mgm./kgm.

(b) In order to obtain detoxification of Mapharsen, the following dosages of BAL were required: 40 mgm./kgm. Mapharsen, which kills 45% of the mice, is completely detoxified by 2 x 40 mgm./kgm. BAL. The MLD₁₀₀ of Mapharsen—60 mgm./kgm.—gives lower mortality figures by increasing the BAL dose from 2 x 40 to 2 x 80 mgm./kgm. Above this BAL dose the mortality rate rises again, due to combined toxicity. Similar results are obtained with higher Mapharsen doses. (Table 1)

II. *Influence of BAL on the therapeutic effect of Mapharsen.* (a) Mapharsen alone, by single subcutaneous treatment in doses of 5 mgm./kgm. sterilized the majority of the mice (71%); 2.5 mgm./kgm. sterilized 30%. Lower doses—1.2 mgm./kgm.—cleared the trypanosomes from the blood, but relapses followed within 2-3 days.

TABLE 1
Detoxification of Mapharsen by BAL

MGm./KGm. OF BAL (GIVEN TWICE)	MAPHARSEN DOSE					
	40 mgm./kgm.*		60 mgm./kgm.		70 mgm./kgm.	
	No. mice	% died	No. mice	% died	No. mice	% died
0	53	45.5	36	100	—	—
20	10	20	5	100	—	—
40	10	0	25	52	—	—
60-70	10	0	65	54	20	60
80	10	0	25	40	5	80
100	10	20	35	74	25	56
125-150	—	—	10	100	20	90

* 50 mgm./kgm. Mapharsen gave 86.7% mortality (53 mice).

(b) The therapeutic effect of combined treatment with various doses of BAL and Mapharsen is presented in Table 2.

A minimal dose of 2 x 0.31 mgm./kgm. BAL is needed to interfere with the sterilizing activity of 5 mgm./kgm. Mapharsen. Combined treatment with these doses gives only a 33% cure, though the trypanosomes disappear temporarily in 83% of the mice. A higher dose of BAL—2 x 0.62 mgm./kgm.—reduces the sterilization to 15%, and about 62% of the group is cleared of parasites for some time. Treatment with 2 x 1.25 mgm./kgm. of BAL entirely removes the sterilizing power of 5 mgm./kgm. of Mapharsen, and more than half of the mice are never cleared of parasites.

Thus, much more BAL is required to abolish the *trypanocidal* than the *curative* (sterilizing) activity of Mapharsen (Table 5).

With increasing BAL doses—above 1.25 mgm./kgm.—there is a delay in clearing the blood of trypanosomes; in most cases the parasites are still present 2 days after treatment, while with Mapharsen alone the majority of the mice becomes negative within a few hours (Table 3).

With higher Mapharsen doses used for therapy a similar picture is recorded, but the BAL doses required to obtain it are higher. For instance, the therapeutic effect of 20 mgm./kgm. Mapharsen is not influenced by as much as 2×5

TABLE 2
Interference of BAL with the therapeutic effect of Mapharsen

MG/M. OF MAPHARSEN		MG/M. OF BAL (GIVEN TWICE)									Control Maph. Only
		60	40	20	10	5.0	2.5	1.25	0.31-0.62	0.08-0.15	
20	A*	2/4	3/3	3/4	5/5	2/2					
	B†	0/4	0/3	0/4	4/5	2/2					
10	A	0/5	0/5	0/3	6/10	9/10	4/4	5/5			9/9
	B	0/5	0/5	0/3	1/10	0/10	2/4	3/5			9/9
5.0	A	2/16	0/10	0/5	3/5	1/5	10/20	6/13	21/29	16/16	33/35
	B	0/16	0/10	0/5	0/5	0/5	0/20	0/13	8/29	12/16	25/35
2.5	A	0/11	0/5	0/5	0/5	0/5	1/5	5/5			15/17
	B	0/11	0/5	0/5	0/5	0/5	0/5	0/5			5/17

* A = Number of mice cleared/Number of mice treated.

† B = Number of mice sterilized/Number of mice treated.

TABLE 3
Trypanocidal activity of 5 mgm /kgm. Mapharsen under influence of various BAL doses

BAL (MG/M. x 2)	NO. TRYPANOSOME POSITIVE MICE/NO MICE ALIVE AFTER DAYS						% MICE ALIVE AFTER 30 DAYS
	1	2	5	8	12	30	
5	5/5	4/5	1/1	/0			0
2.5	18/20	10/20	11/14	9/9	/0		0
1.25	11/13	7/13	5/9	3/6	3/4	0/1	7.7
0.62	9/13	5/13	4/9	3/7	2/5	0/2	15
0.31	6/16	3/16	4/14	4/10	3/9	0/6	37
0.08-0.15	0/16	0/16	0/16	0/16	0/16	0/12	75
0	5/35	2/35	0/33	0/33	4/33	0/25	71

TABLE 4

Duration of the protective effect of 40 mg./kg. BAL against intravenous Mapharsen (50 mg./kg.)

	MAPHARSEN INJECTION FOLLOWS AFTER					
	½ minute	½ hr.	1 hr.	1½-2 hrs	3-4 hrs.	5 hrs.
No. of mice	20	10	10	20	30	10
Mortality . .	10%	60%	50%	85%	73%	100%

mgm./kgm. of BAL: 100% of the mice are sterilized. Only with 2×10 mgm./kgm. is there a delay in clearing of the parasites noticed. With 2×20 mgm./kgm. of BAL the blood of the majority of the mice (75%) is still cleared, though relapses follow which kill these animals in an average of 11.3 days after treatment.

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	No. mice	% died	No. mice	% died	No. mice	% died
0	53	45.5	36	100	—	—
20	10	20	5	100	—	—
40	10	0	25	52	—	—
60-70	10	0	65	54	20	60
80	10	0	25	40	5	80
100	10	20	35	74	25	56
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20	A*	2/4	3/3	3/4	5/5	2/2					
	B†	0/4	0/3	0/4	4/5	2/2					
10	A	0/5	0/5	0/3	6/10	9/10	4/4	5/5			9/9
	B	0/5	0/5	0/3	1/10	0/10	2/4	3/5			9/9
5.0	A	2/16	0/10	0/5	3/5	1/5	10/20	6/13	21/29	16/16	33/35
	B	0/16	0/10	0/5	0/5	0/5	0/20	0/13	8/29	12/16	25/35
2.5	A	0/11	0/5	0/5	0/5	0/5	1/5	5/5			15/17
	B	0/11	0/5	0/5	0/5	0/5	0/5	0/5			5/17

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Trypanocidal activity of 5 mgm./kgm. Mapharsen under influence of various BAL doses

BAL (MGm./KGm. $\times 2$)	NO. TRYPANOSOME POSITIVE MICE/NO. MICE ALIVE AFTER DAYS						% MICE ALIVE AFTER 30 DAYS
	1	2	5	8	12	30	
5	5/5	4/5	1/1	/0			0
2.5	18/20	10/20	11/14	9/9	/0		0
1.25	11/13	7/13	5/9	3/6	3/4	0/1	7.7
0.62	9/13	5/13	4/9	3/7	2/5	0/2	15
0.31	6/16	3/16	4/14	4/10	3/9	0/6	37
0.08-0.15	0/16	0/16	0/16	0/16	0/16	0/12	75
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mgm./kgm. of BAL: 100% of the mice are sterilized. Only with 2×10 mgm./kgm. is there a delay in clearing of the parasites noticed. With 2×20 mgm./kgm. of BAL the blood of the majority of the mice (75%) is still cleared, though relapses follow which kill these animals in an average of 11.3 days after treatment.

III. *Experiments with intravenous Mapharsen injection.* In order to determine whether the conditions of the absorption of Mapharsen might influence these results, we administered BAL subcutaneously, and immediately (in less than $\frac{1}{2}$ minute) afterwards, Mapharsen *intravenously*.

(a) Detoxification. Under these conditions, approximately 30 mgm./kgm. BAL are required to detoxify the MLD₁₀₀ of Mapharsen, 50 mgm./kgm. The protection afforded by the subcutaneously administered BAL against intravenous Mapharsen is maximal immediately after injection (90% protection); decreases within 45 minutes (40% protection), and disappears almost entirely within 1½-3 hours (15-27% protection). This prompt appearance of the protective effect

TABLE 5

BAL/Mapharsen ratios required for interference with toxic and therapeutic Mapharsen doses

MAPHARSEN MG./KG., S.C.	BAL MG./KG. (TOTAL) S.C.	BAL/MAPHARSEN
Partial detoxification		
70	190	2.7
60	80	1.33
40	40	1.0
Loss of sterilizing activity		
20	30-40	1.5-2
10	5-10	0.5-1
5	0.62	0.12
5*	0.08	0.016
Loss of trypanocidal activity		
20	120	6.0
10	40	4.0
5	2.5	0.5
5*	2.5	0.5

* Intravenous.

indicates that BAL administered subcutaneously in oil to mice is very rapidly absorbed (Table 4).

(b) Therapeutic experiments. Infected mice were treated with varying doses of BAL, followed by 5 mgm./kgm. intravenous Mapharsen, a dose which cures without relapses 85% of the mice. When treatment is preceded by 2.5-10.0 mgm./kgm. BAL, this Mapharsen dose becomes completely ineffective: the trypanosomes are not affected and the animals die (15/15), though a little later than the controls. With lower doses of BAL, 0.62-1.25 mgm./kgm., the trypanocidal effect is only slightly decreased, but the curative activity disappears completely. The trypanosomes disappear within 24 hours from the blood stream of the majority of mice (15/20), but a fatal relapse follows in almost all mice (13/15) within an average of 7 days. By decreasing the dose of BAL to 0.15-0.31 mgm./

kgm., the trypanocidal effect apparently is no longer influenced, though a fatal relapse follows in 16 out of 20 mice within an average period of 11 days. The same phenomenon is evident even with lower doses, except the period of relapse is more delayed. Five mice treated with 0.08 mgm./kgm. BAL were cleared completely of the parasites within 18 hours following the intravenous Mapharsen injection, but they relapsed (4/5) within an average of 13 days. Only doses of BAL as low as 0.04 mgm./kgm., or lower, are without effect on the curative activity of 5 mgm./kgm. Mapharsen.

These experiments also show very convincingly that much higher doses of BAL are required to interfere with the *trypanocidal* than with the *curative* activity of Mapharsen. With the dosage of Mapharsen used, the interference with the *curative* activity takes place with $\frac{1}{30}$ th of the BAL dose required to abolish the trypanocidal effectiveness.

CONCLUSIONS

Effective doses of BAL against Mapharsen caused either a prolongation of the time necessary to clear the blood, or reduced the percentage of sterilized animals, or actually prevented the disappearance of trypanosomes. Often in the same group of mice all three processes were visible, to varying extent, according to the BAL-Mapharsen dose relation.

Each phase of our experimental work points to the conclusion that the *curative* (sterilizing) action of Mapharsen is influenced much more readily by BAL than the *trypanocidal* action, while the toxicity, on the other hand, is the least affected.

The doses of BAL giving inhibition of curative, trypanocidal, and toxic activities of Mapharsen are contained in Table 5.

To interfere with toxic Mapharsen doses, 1.0-2.7 times more BAL than Mapharsen is required, while the *curative* effect disappears with doses of BAL as small as $\frac{1}{4}$ - $\frac{1}{2}$ of the Mapharsen weight.

Expressing these results in the classical form of "therapeutic index", one could say that this is lower for the combined treatment than for Mapharsen alone, since the curative activity is more reduced than the toxicity by the same proportional dose of BAL.

Yet, we are not in a position to say whether BAL influences differently the specific biological action of Mapharsen on the host and on the parasite. It appears that the (relative) dose of BAL needed for inhibition increases with the Mapharsen dosage. Since the therapeutic effect begins at a lower dose than the toxic one, it is possible that the *quantitatively* different influence of BAL on the two biological effects depends only upon the difference of dose range on which BAL operates.

With increased Mapharsen doses relatively more BAL is needed for interference with both toxic and therapeutic activity. Perhaps this might be explained by assuming that interference begins when the arsenical content descends below a certain threshold level; to descend to this level a proportionately higher fraction of the arsenical has to be neutralized, or eliminated, when higher doses are given.

That BAL so readily reverses the *curative* effect of Mapharsen in contrast to its *trypanocidal* activity, might be explained, at least in part, by observations made

by Eagle, Magnuson and Fleischman (4) and by Riker and Rosenfeld (7), concerning the metabolism of the arsenical under the influence of BAL.

BAL increases (7), in the first hours after the Mapharsen injection, the As concentration in the blood, utilizing all the As available to the tissues. This increased concentration of As in the blood might account for the conservation of trypanocidal activity. It is not known in which form Mapharsen appears in the blood after BAL treatment, but even assuming that it is completely bound in the form of thioarsenite, some of its immediate effect should be conserved, since this compound is active, (though less than Mapharsen), according to the recent publication of Friedheim (6).

The rapid elimination of the arsenical from the organism (4) would be responsible for the more manifest decrease in curative activity. Not sufficient arsenical remains in the host organism—and/or the duration of effect is not prolonged enough—to induce biological cure. In fact, to some extent, the decrease of therapeutic activity appears in similarity to the decrease in the dose of arsenical, for instance, the therapeutic action of the combined treatment with 5 mgm./kgm. Mapharsen and 2×0.31 mgm. of BAL can be compared to that of 2.5 mgm. of Mapharsen alone.

It should be kept in mind, however, that all therapeutic phenomena in trypanosome infection are accompanied by immune reactions (8, 9).

The immune response following Mapharsen therapy might be influenced by the changed rate of disappearance of the trypanosomes under the influence of BAL. The interpretation on this basis of the discrepancy between initial clearance of the trypanosomes and high relapse rate warrants further investigation.

SUMMARY

The therapeutic activity of Mapharsen, under the influence of BAL, has been studied in relation to its toxicity in mice infected with *T. equiperdum*. It was found that: 1) The curative activity of the arsenical is inhibited by much lower doses of BAL than the trypanocidal activity. 2) The therapeutic activity of the arsenical is inhibited by relatively lower doses of BAL than the toxicity, as measured by the mortality figures. 3) In general, the higher the dose of the arsenical the more BAL proportionately is required for inhibition. 4) The extremely fast decrease in the curative action of Mapharsen, when given in conjunction with BAL, precludes the possibility of this form of combined chemotherapy.

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CURARIFORM ACTIVITY OF QUATERNARY AMMONIUM IODIDES DERIVED FROM CINCHONA ALKALOIDS¹

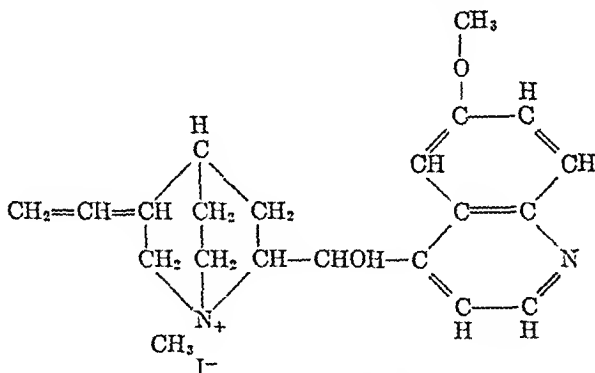
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Rosenstein (1), applying the premise of Brown and Fraser (2) that onium ions have curariform activity, discovered the lissive action of einchonine methochloride and quinine methiodide. Harvey (3) studied quinine methochloride in cats and Bennett (4) applied his findings clinically. Chase, Lehman, and Rickards (5) thoroughly investigated quinine methochloride and its higher N-alkyl homologs through quinine hexyl bromide. We have compared quinidine methiodide,² quinidine ethiodide, einchonine methiodide, einchonine ethiodide, cinchonidine methiodide, quinine methiodide, and quinine ethiodide.

The compounds used were prepared in this laboratory by accepted procedures and analyses and properties checked with the literature (6).



The formula given for quinine methiodide is identical with that for the isomer, quinidine methiodide. The einchonine and einchonidine compounds differ only in the absence of the methoxy group. The ethiodides differ in the replacement of the nitrogen methyl group by an ethyl group. Since the iodide ion is of no significance in the physiological activity of these agents, all doses have been computed on the weight of ammonium ion contained.

The relative activity was first determined in 340 albino rats (200-300 Grams). Five-tenths per cent solutions were injected intraperitoneally and the paralytic

¹ Part of the material in this paper was presented before the American Society of Pharmacology and Experimental Therapeutics, Federation meetings, Chicago, 1947. See *Federation Proceedings* 6: 354, 1947.

² These compounds might be more correctly termed "N-methyl quinidinium iodide" etc., but long established usage indicates the name "quinidine methiodide".

and lethal doses determined (see table 1). The quinidine and cinchonidine compounds are most active and most toxic, the quinine compounds produce better lissive action with less fasciculation and anoxic convulsive movements, and the cinchonine compounds are least active. The relative safety margin of these compounds is very small. It is not possible to determine the corresponding paralytic or lissive doses for the rat with partially purified Chondodendron tomentosum extracts ("Intocostin") or with pure d-tubocurarine chloride, since respiratory paralysis occurs simultaneously with paralysis of the hind limbs.

Since the relative effects obtained in the rat might be an anomaly dependent on the test object involved, we determined the activity in the rabbit. The dose producing head drop by Holaday's method (4) and the lethal intravenous dose

TABLE 1
Comparative activity of cinchona alkoidides

	RABBIT		RAT		CAT
	Head drop 50*	Lethal dose 40-60%	Paralytic dose 45-55%†	Lethal dose 45-55%	Gastrocnemius muscle paralysis‡
	<i>Dosage in mgm /kgm.</i>				
Quinine methiodide . . .	5	7	18	23	3
Quinine ethiodide . . .	6	9	18	23	—
Quinidine methiodide .. .	2	3.5	10	13	1
Quinidine ethiodide . . .	4	6	23	28	—
Cinchonidine methiodide	3	4.5	10	13	1.5
Cinchonidine ethiodide	7	10	20	25	—
Cinchonine methiodide	3	4	15	20	3
Cinchonine ethiodide	10	13	35	40	—
d-Tubocurarine chloride . . .	0.12	0.35	—	0.25	0.05
Intocostin	1.0	1.9	—	4.0	0.25

* Dose producing head drop lasting a minimum of three minutes in three of six rabbits. Lethal doses based on groups of five rabbits.

† Dose producing paralysis of hind legs for a minimum of three minutes in groups of twenty rats.

‡ Paralysis for three minutes after intravenous injection.

in 40 rabbits were determined. It was found that rabbits which survived the effects of the agents could be used repeatedly with the same apparent quantitative response. Comparable results in the rabbit with the quinine compounds have been obtained by the other workers (5, 7).

The cat gastrocnemius preparation was paralyzed by the systemic administration of these agents at 1-3 mgm./kgm. The quinidine compounds are most potent, then cinchonidine, quinine and cinchonine least. Quantitatively similar effects can be produced by $\frac{1}{2}$ this amount of Intocostin and $\frac{1}{20}$ of d-tubocurarine chloride. Like crude strychnos curare, these cinchona derivatives produce an accompanying, transient fall in blood pressure that is incompletely antagonizable by atropine, and is not related to the oxygenation of the animal. For an

equal fall in blood pressure, as illustrated by the figure, Intocostrin is far superior in lissive effect to these cinchona derivatives.

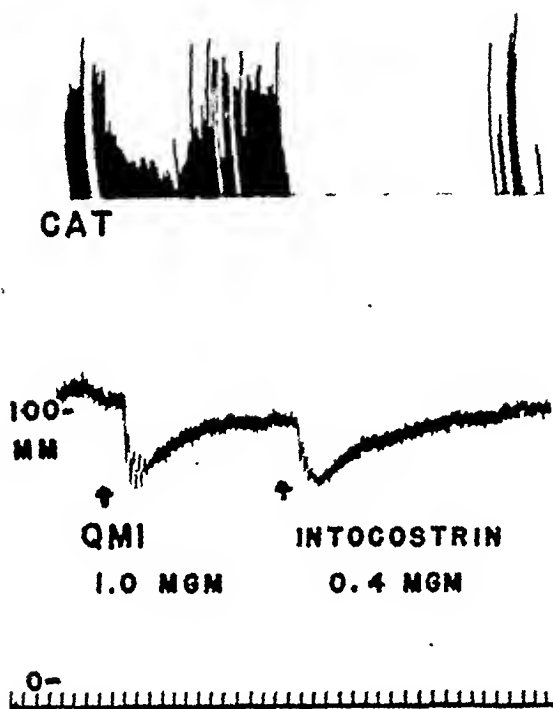


FIG. 1. 2.9 kgm. male cat. 270 mgm./kgm. Na Barbitol intraperitoneally. 1 mgm./kgm. atropine sulfate intravenously. Mechanical oxygenation. Gastrocnemius muscle recording through weighted tendon. Peripheral end of cut sciatic nerve stimulated with a motor driven interrupter for one tenth second with 6 volts 60 cycle halfwave every ten seconds. Blood pressure in mm Hg. Time line in minutes.

SUMMARY

The curariform activity of quinidine methiodide, quinidine ethiodide, cinchonine methiodide, cinchonine ethiodide, cinchonidine methiodide, and cinchonidine ethiodide in comparison with quinine methiodide and quinine ethiodide was determined in rats, rabbits, and cats. The quinidine and cinchonidine compounds are most active and most toxic, the quinine compounds produce better lissive action with less fasciculation, and the cinchonine compounds are least active.

Acknowledgment. We are grateful to H. S. Newcomer, E. R. Squibb & Sons, New York for the Intocostrin, and to D. L. Tabern, Abbott Laboratories, North Chicago, Illinois, for the d-tubocurarine chloride used in this study.

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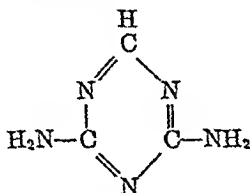
DIURETIC ACTION OF FORMOGUANAMINE IN NORMAL PERSONS

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We recently reported on several new diuretics more potent than urea (1, 2, 3, 4). They contain several $\text{=N}-\overset{\text{H}}{\underset{\text{||}}{\text{C}}}-\text{N=}$ groups in the molecule. It appears that formoguanamine is 347 times as active as urea in the rat, and 145 times in



the dog (mol per mol). Thus, the diuretic dose range of formoguanamine was 7.5 to 30 mg./kg. in comparison with 700 to 2500 mg./kg. for urea, assayed on the same dog. NaCl is excreted in considerable amounts in the urine under the influence of the drug. Previous data (4) suggesting that formoguanamine is essentially free from side actions were supplemented by new experiments on dogs and rabbits. Functional tests and histological examinations were made in the course of a prolonged daily administration of suitable diuretic doses of formoguanamine.

EXPERIMENTS ON DOGS. Twelve female dogs were divided into two groups of similar weight. Several weeks before the beginning of the experiments, the external urethral opening was exposed through a Falcx incision in order to facilitate catheterizing. One group served as controls; the other, after a preperiod of extended observation, were fed daily 15 mg./kg. formoguanamine, a definitely diuretic dose, six times weekly for 12 weeks during a standard food regime. Body weight, temperature, hemoglobin and blood counts were recorded. Bromsulphalein tests were made as a check of liver function. The functions of the kidney were tested by three methods; 1. Water test: The dogs fasted over night were hydrated by oral administration of 40 ml./kg. of tap water, and after three hours the bladders were drained and the animals were again fed the same amount of water. The excretion of urine in 3 hours was estimated in terms of per cent of the "second water". Normally these figures are between 70 and 100 per cent. 2. Phenolsulphonphthalein test: The fasted dogs were slightly hydrated by oral administration of 10 ml./kg. of tap water, and after 40 minutes 6 mg. phenolsulphonphthalein in 1 ml. fluid was injected intramuscularly. The amount of dye excreted in the urine after 70 and 120 minutes was assayed in terms of per cent of the amount injected. Sixty per cent or more are normally excreted in 120 minutes. 3. Diuretic effect of formoguanamine: The effect of the first dose of 15 mg./kg. drug given in a capsule was determined in the dogs which had fasted over night and had only 50 ml. of water in the cage. In a preperiod of 2 to 3 hours—when no water or food was given—the basic urinary excretion per hour was determined. Then the 5 hour

urinary excretion under the influence of the drug was measured, and the ratio, drug period/5x preperiod, was used as the expression of diuretic action; at a ratio of 1 no diuretic effect is apparent.

As shown in detail in figures 1 and 2, the mean change in body weight in the formoguanamine group was -1 per cent during the dosing period, whereas the change in the control group was +6 per cent. No significant influence of the drug upon body temperature, blood counts or blood picture could be detected, nor did the bromsulphalein test reveal any deterioration of this liver function.

Neither the water excretion nor the phenolsulfonphthalein excretion of the kidneys was consistently influenced in the test period with the exception of one dog (no. 21). The diuretic effect of the formoguanamine itself was approximately as strong after the 72 doses as in the first diuretic experiment, again with the exception of dog no. 21. All the dogs were autopsied by Dr. F. I. Dessau and the important organs were histologically examined. No significant changes were recorded. In dog no. 21 tubular distension and cortical scars were seen in the kidneys. However, similar scars were found also in one of the six control dogs (no. 68); other incidental deviations from normal were scattered and almost evenly distributed between the controls and the dogs fed formoguanamine.

EXPERIMENTS ON RABBITS. Twelve male rabbits were divided into two equal groups. One served as control, the other rabbits were daily fed 20 mg./kg. formoguanamine. Both groups were under the same food regime. The formoguanamine rabbits excreted large amounts of urine of a lighter color than that of the controls. Treatment was continued for 4 weeks six times weekly. The mean change in body weight for the test animals was +14.4 per cent, the change in the control animals +10.5 per cent. No significant change in body temperature was recorded (fig. 3), and no pathological findings which could be related to the treatment were made when the animals were sacrificed and studied macroscopically and histologically at the end of the test period (Dr. Dessau).

EXPERIMENTS ON HUMANS. After the diuretic activity of formoguanamine on rats, rabbits and dogs was established and no untoward side action was apparent in acute or chronic experiments, we proceeded to test the drug on normal human subjects. Eight healthy men volunteered for the test. The procedure chosen approximated that of the animal experiments as closely as possible. The persons were fasting over night and did not receive food until the end of the experiments. The test period, by necessity, covered only 2-3 hours before and the 4 hours following the administration of the drug. In the first, preliminary series of experiments, no fluid intake except 10-20 ml. of water accompanied or followed the oral administration of the drug in capsules. The results are given in fig. 4. All four persons after a latent period of one hour, reacted to 8.5 to 10 mg./kg. formoguanamine with a varying increase of urinary excretion which, in two persons, had not returned to that of the preperiod after four hours. After the experiment, the persons had a freely chosen meal. Two subjects had a distinct feeling of discomfort in the lumbar region, were thirsty and continued to void increased amounts of urine. The urines after cooling showed a considerable crystalline precipitate mainly due to *ammelide*, a metabolite of formo-

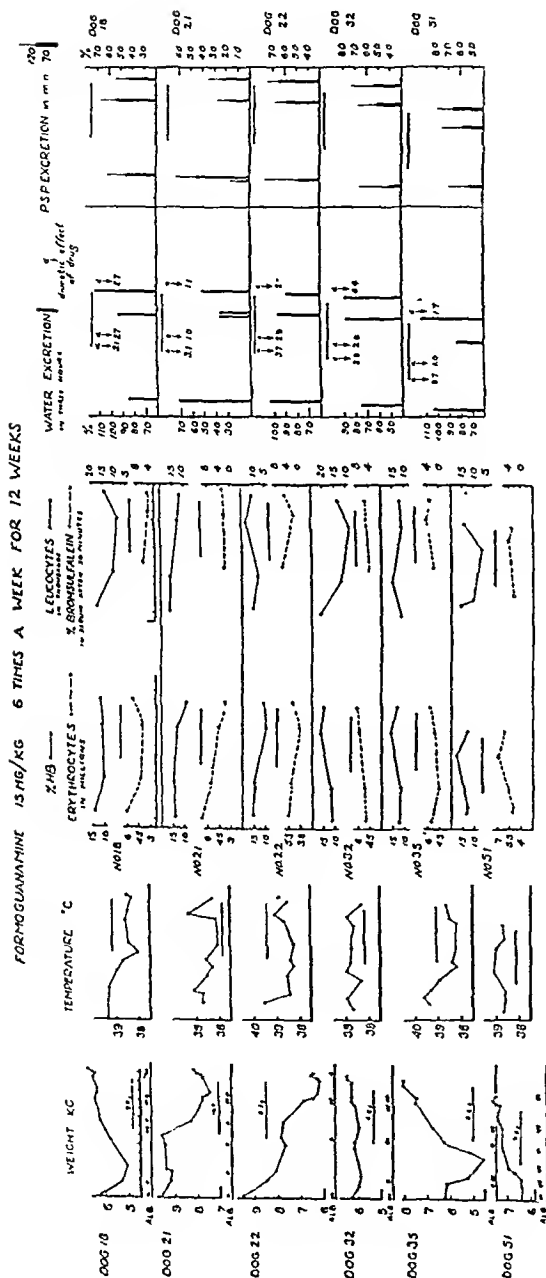


FIG. 1. TESTS IN DOGS FED FORMOGUANAMINE

The test period is marked by the horizontal line, and the total of the drug given in grams by the figures above that line in the weight chart.
 Alb. = urinary albumen.

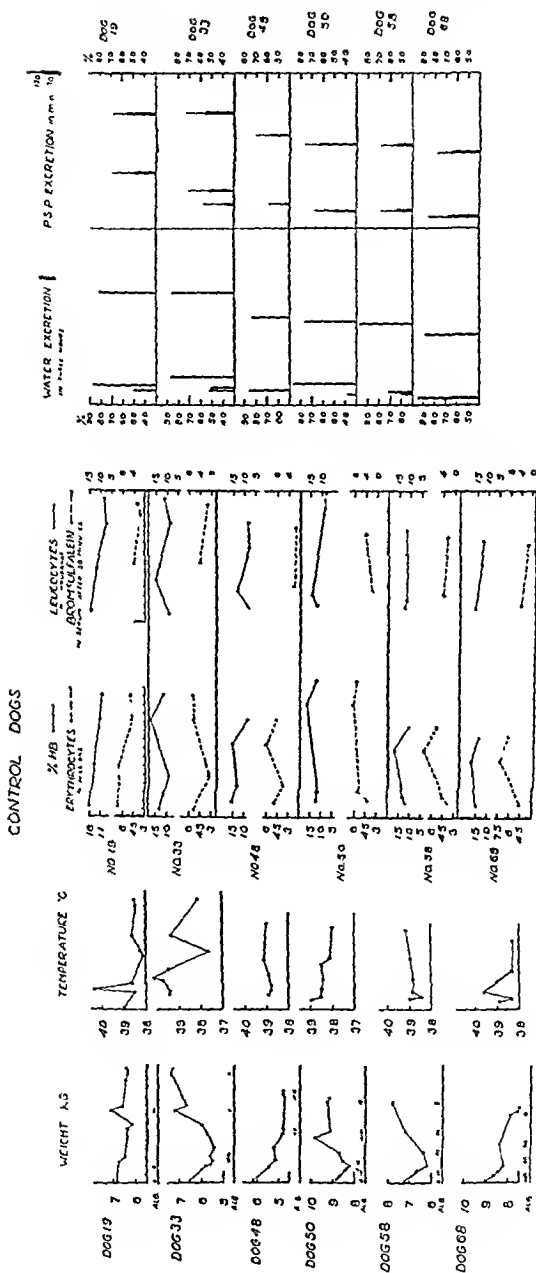


FIG. 2. TESTS IN CONTROL DOGS OVER A PERIOD OF 6 MONTHS

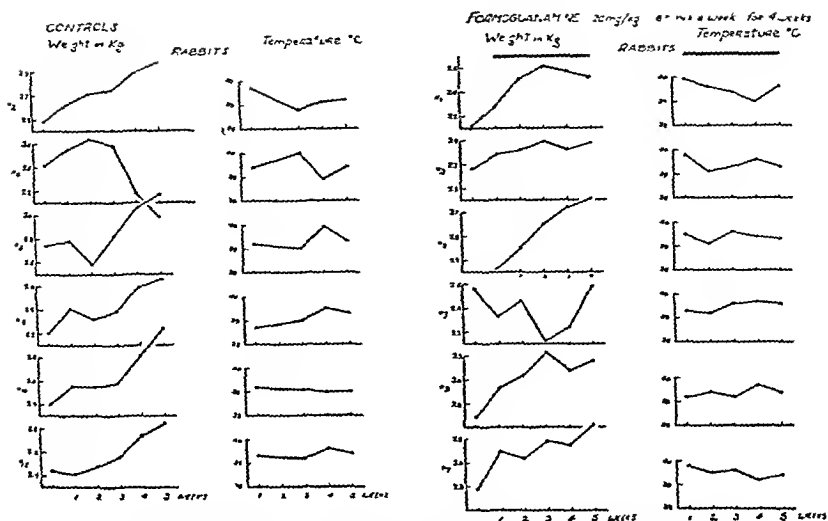


FIG. 3. BODY WEIGHTS AND TEMPERATURES OF RABBITS TREATED WITH FORMOGUANAMINE OVER A PERIOD OF 5 WEEKS, AND OF CONTROL RABBITS

The dosing period is marked by the horizontal line on top of the charts.

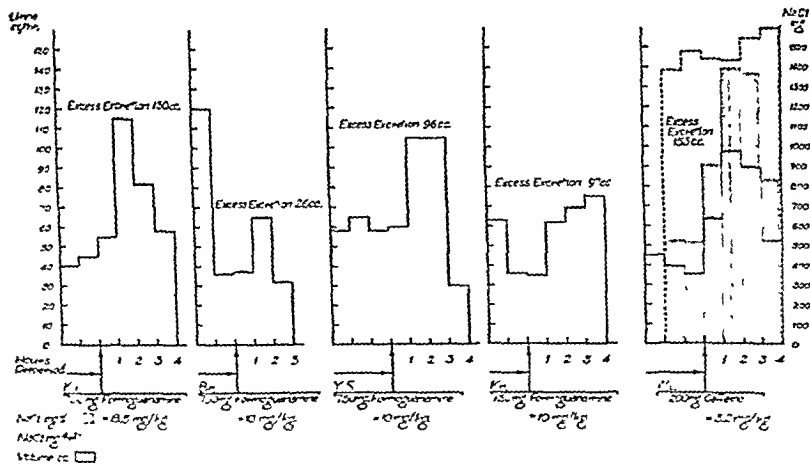


FIG. 4. THE DIURESIS PRODUCED BY FORMOGUANAMINE OR CAFFEINE IN FIVE NORMAL FASTING MEN

guanamine (5); but in the freshly voided urine of 37° C. the crystalluria was mostly insignificant. For comparison, a fifth volunteer took 200 mg. of caffeine under the same experimental conditions, thus duplicating older experiments of Lie (6). Diuresis, somewhat stronger than seen in the formoguanamine ex-

periment, was produced in this individual, and the excretion of chlorides, too, was increased.

A second series of experiments was then undertaken, designed to closer approximate the conditions of excess of tissue water in the edematous patient, and also in order to study the effect of varying doses of formoguanamine upon output of water and chloride. After a preperiod of two hours in which the basic urinary excretion of the persons (overnight fasted) was established, they drank 350 ml. of 0.85% NaCl solution within a few minutes. The hourly urinary excretion and the chloride excretion was followed for 4 hours. About one week later this control experiment was repeated, and in the following weeks formoguanamine doses ranging from 3.5 to 11.0 mg./kg. were taken together with the 350 ml. of saline solution. A group of representative experiments in the same person are given in detail in fig. 5; a summary of the results in all four persons will be found in fig. 6.

It appears that 3.6 to 5.4 mg./kg. of formoguanamine are the diuretic threshold dose, and that 8 to 9 mg./kg. are a highly effective dose: In 4 hours the total of the saline intake is eliminated. Eleven mg./kg. seems to represent an overdose in normal subjects causing some discomfort in the lumbar region, thirst and prolonged diuresis. In this instance, the loss in water and salt became evident when a second saline dose was given without drug on the following day. The excretion of fluid as well as of NaCl was extremely low; almost all of it was retained in the body.

The comparison of formoguanamine with xanthine diuresis is particularly interesting. In the case of one individual (W. L.), 1.6 and 3.1 mg./kg. respectively of caffeine produced diuretic effects similar to those of 4.6 and 7.8 mg./kg. formoguanamine and in proportion to the dose. However, the effects of similar doses of caffeine were just recognizable in the other subject (E. S.) who had reacted well to formoguanamine; also the high dose of 3 gm. theobromine with sodium salicylate (diuretin) produced only a slight diuretic effect in E. S.

Discussion. The data on animal experimentation reported here and in a previous publication (4) demonstrate the usefulness of these methods for preliminary investigational work on diuretics. They provide evidence that the animal responds in an essentially similar manner as does man. Our data on the effect of formoguanamine in human subjects prove that this compound evokes diuresis and chloruresis in man as well as it does in rats, rabbits and dogs. The xanthine diuretics, whose effect is subject to great individual variation in rats and dogs (1, 2, 4), vary also greatly in individual efficacy in normal human beings.

E. Lie (6), and Blumgart and associates (7), demonstrated earlier that trials on normal persons are of value in bridging the gap between the normal animal and the edematous patient. They showed that caffeine, diuretin, metaphylline, merbaphen and salyrgan are diuretically active both in normal and in edematous persons. Our procedure of testing diuretics on fasting subjects offers a relatively simple experimental set-up which yields significant results. It consists in giving a standard amount of saline solution simultaneously with the varying doses of the drug, and comparing both water and NaCl excretion

with the excretion without drug in the same person. Hospital conditions obviously would permit the further extension and variation of experimental studies.

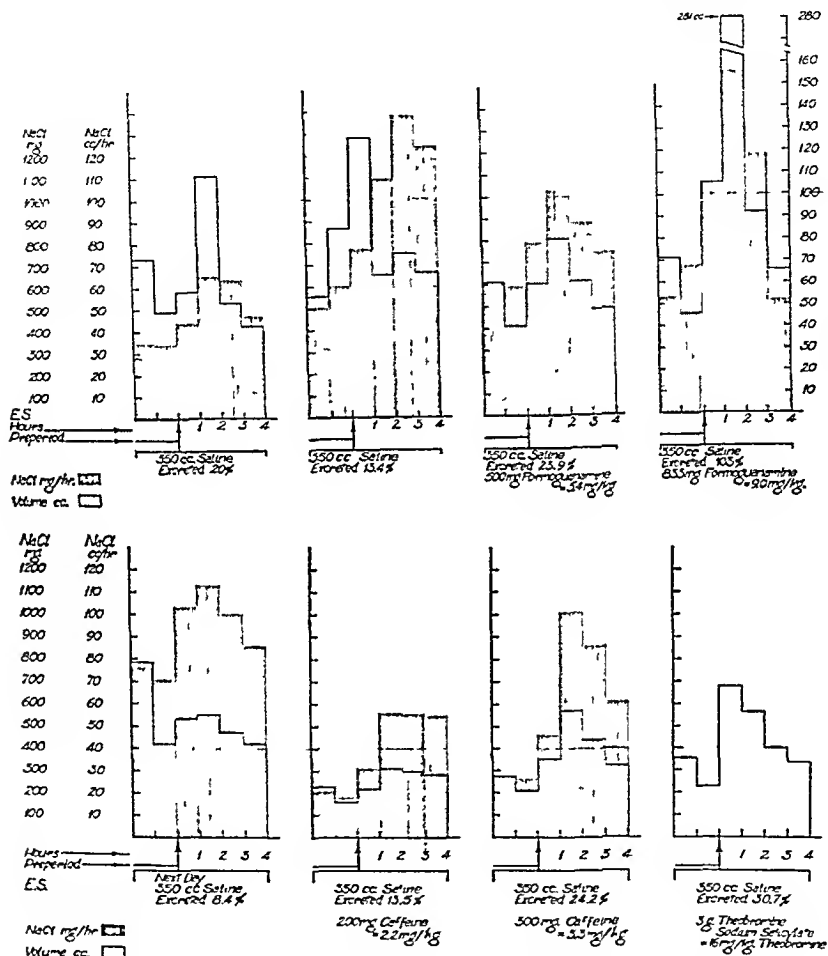


FIG. 5. EXPERIMENTS ON A NORMAL FASTING PERSON SHOWING THE HOURLY URINARY EXCRETION AND CHLORIDE EXCRETION AFTER SALINE INTAKE WITH AND WITHOUT FORMOGUANAMINE, CAFFEINE AND DIURETIN.

Attention may be directed to the interesting fact that in the dog 7.5 to 30 mg./kg. was found the appropriate diuretic dose range of formoguanamine, and that correspondingly in the normal human 8 to 9 mg./kg. were diuretically highly effective. Thus assay methods for diuretics are available, which make it possible to relate a dose range effective in animals to dosage in man.

The therapeutic usefulness in patients and the field of application of a new diuretic, as formoguanamine, will obviously need systematic clinical investigation. In this respect, it may be noted that H. Ludwig (8) demonstrated in the

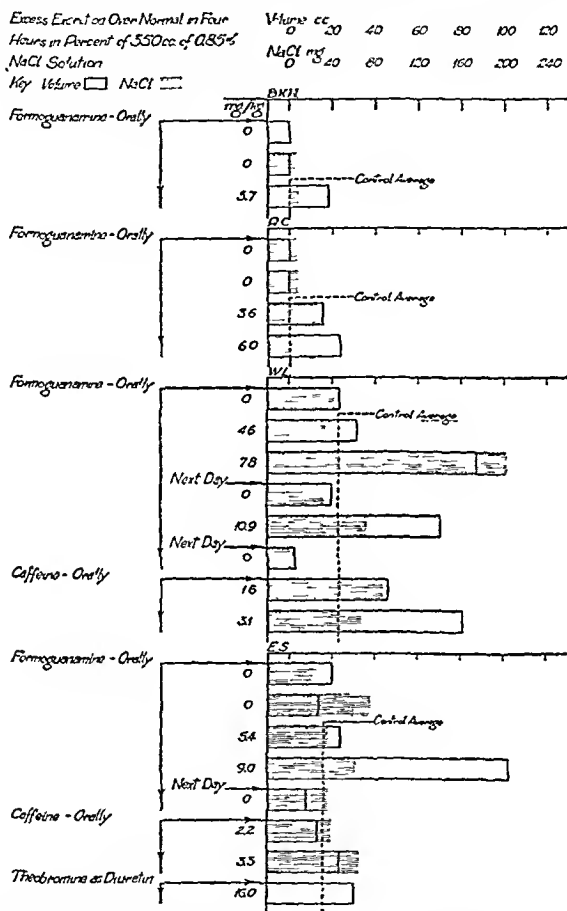


FIG. 6. DIURETIC EFFECTS IN FOUR HOURS ON FOUR NORMAL FASTING SUBJECTS AFTER VARYING DOSES OF FORMOGUANAMINE OR OF CAFFEINE, GIVEN SIMULTANEOUSLY WITH STANDARD SALINE DOSE

first clinical evaluation of formoguanamine that it is active and well tolerated in edematous patients as well as in convalescent persons.

SUMMARY

1. Diuretic doses of 15 mg./kg. of formoguanamine were fed to a group of six dogs 72 times each, and 24 doses of 20 mg./kg. to a group of six rabbits without producing toxic effects.

2. In agreement with previous assays in rats and dogs, formoguanamine produces a significant diuresis and chloruresis in normal fasting persons in doses between 4 and 9 mg./kg.

3. Under standard conditions of test in human beings—administration of 350 ml. of 0.85% NaCl solution together with the diuretic—8 to 9 mg./kg. of formoguanamine removed 90 to 100 per cent of the introduced fluid in four hours.

4. Doses above 9 mg./kg. may produce thirst, prolonged diuresis and feeling of discomfort in the lumbar region.

5. Formoguanamine proved to be more consistent in diuretic activity than the xanthine diuretics under comparable conditions.

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OBSERVATIONS ON THE PHARMACOLOGY OF THE ISOMERS OF HEXACHLOROCYCLOHEXANE

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I. Introduction. In 1942 the Hawthorndale Laboratories, England, tested a series of chlorinated cyclohexanes for their insecticidal activity against flea beetles. Among the compounds tested was a sample of crude benzene hexachloride ("666" or 1, 2, 3, 4, 5, 6 hexachlorocyclohexane). This crude material exhibited outstanding activity. After the isolation of four spatial isomers from the crude material, it was found that the isomer designated as gamma (gammexane) possessed the greatest insecticidal properties (1).

The oral, subcutaneous and percutaneous toxicity of the gamma isomer in various animals has been reported by Cameron and Burgess (2). The symptomatology and autopsy findings in gammexane poisoned animals was also discussed by these authors. Aside from the importance of the poisoning hazard in man and domestic animals incident to the use of potent insecticides, considerable pharmacological interest attaches to convulsant halogenated hydrocarbons, because halogenated hydrocarbons are generally regarded as central nervous system depressants. The stereo-isomers of 1, 2, 3, 4, 5, 6 hexachlorocyclohexane offer a unique case for observations on the relationship between chemical structure and pharmacological activity, inasmuch as the isomerism involves space arrangements of identical substituents. The following report is based on a study of the effects of hexachlorocyclohexane stereo-isomers in rabbits and dogs.

II. EXPERIMENTAL. a. *The intravenous toxicity of the mixed isomers of "666" in rabbits.* Preliminary experiments indicated that the toxicity of the commercial mixture was not as great as its gammexane content required. To eliminate the possibility that the undetermined fraction of the commercial mixture altered the activity of the gammexane portion to a significant extent, the four pure isomers¹ were mixed in proportions approximating those in the crude material (alpha 70%, beta 5%, gamma 12%, delta 7%, unaccounted 6%), and the pharmacological properties of the resulting preparation was studied in dogs and rabbits.

The solubility of the various isomers of 666 imposes limitations on the pharmacological study by the intravenous route. However, satisfactory preparations were made by emulsification of oil solutions. The following quantities of isomers were dissolved in 50 cc. of warm peanut oil, 5.833 grams of alpha, 0.416 grams of beta, 1.0 gram of gamma and 0.583 grams of delta (70:5:12:7). A homogenizer was then used to emulsify 6.25 cc. of the warm oil solution with sufficient 1% soy bean lecithin solution in saline to make 25 cc. One cc. of resulting emulsion, therefore, contained 5 mgm. of the gammexane and the other isomers in the proportions contained in crude hexachlorocyclohexane. Since crystallization occurs on standing in the oil solution, the emulsion must be freshly prepared and frequently homogenized. A 10 cc./kgm. dose of a control emulsion containing no active agent killed one rabbit of a group of 10. Death apparently resulted from lung edema produced by oil embolism. No symptoms were noted in the other rabbits.

¹ We are indebted to Dr. V. S. Sconce of the Hooker Electrochemical Company for the samples of pure isomers used in the study.

Following the intravenous administration of emulsion containing the gammexane to rabbits, symptoms are observable within two minutes. The animals became restless and appeared apprehensive. Respiration was increased in depth and frequency and blepharospasm appeared. Generalized muscle tremors developed and strong convulsions rapidly ensued. During the convulsions respiration ceased and the heart rate fell to low levels; cardiac arrest often resulted. Examination of the heart at such a time showed complete systole of the left ventricle, with auricular diastole. In many cases after cardiac and respiratory failure, it was possible to resuscitate the rabbits by artificial respiration and cardiac massage by gentle intermittent pressure on the thorax. Animals which survived this initial episode exhibited intermittent convulsions between which they remained prostrate. Tremors disappeared but hyperpnea persisted. If death did not occur within one hour, complete recovery usually followed.

The toxicity of this preparation to rabbits is shown in Table I. A detailed discussion of the crude 666 is omitted from this report since its toxicity and pharmacological activity are very similar to those exhibited by the mixed isomer preparation which we have described in this report.

TABLE I
The intravenous toxicity of the mixed isomers of "666" in rabbits

PROPHYLACTIC DOSE OF MIXED ISOMERS	DOSE OF GAMMEXANE CONTAINED IN ISOMER MIXTURE	MORTALITY
mgm./kgm.	mgm./kgm.	
47	6.0	0/1
94	12.0	2/14
141	18.0	2/6
188	24.0	2/2
235	30.0	1/1

b. *Toxicity of the gammexane by intravenous administration.* One volume of 10% solution of gammexane in peanut oil was emulsified with 9 volumes of soy bean lecithin solution in physiological saline. Following the intravenous administration of the emulsion to rabbits the symptoms described for mixed isomers were noted. It should be mentioned that the convulsions produced by gammexane are primarily of high central origin because atropinized spinal cats exhibit such convulsions above the area of transection but little activity is observed caudal to the transection. The toxicity of pure gammexane is shown in Table II.

From a comparison of Tables I and II it may be noted that while 6 mgm./kgm. of the pure gammexane intravenously produced a 100% mortality that 12 mgm./kgm. of this isomer produced only a 14% mortality in the presence of the other isomers. Two possible explanations for this phenomenon were considered: 1) a direct physiological antagonism between the various isomers may exist, or 2) less toxic isomers may be denying the gamma compound access to susceptible body cells. Evidence supporting the first hypothesis will be presented below.

c. *The intravenous toxicity of the beta and delta isomers in rabbits.* The great

insolubility of the beta isomer in non-toxic solvents makes accurate determination of its intravenous toxicity very difficult. Large volumes of oil must be administered to obtain adequate dosage. Death from oil embolism results before accurate toxicity figures are obtainable. The delta compound has a solubility similar to that of the gamma compound. However, its relatively low toxicity also requires the administration of large quantities of oil.

Despite these difficulties a striking pharmacological difference between these isomers and the gamma compound was apparent. Following the intravenous administration of 10 to 20 mgm./kgm. of either the beta or delta compound a rapid onset of paralysis was observed. There was an increase in respiration but cardiac action did not appear abnormal. None of the central effects or muscular tremors produced by the gamma compound appeared. The paralysis due to

TABLE II
The intravenous toxicity of gammexane in rabbits

DOSE OF GAMMEXANE mgm /kgm.	MORTALITY
4.0	0/2
4.5	9/11
5.0	4/5
6.0	24/24

TABLE III
Antagonism between the mixed isomers and gammexane in rabbits

PROPHYLACTIC DOSE OF MIXED ISOMERS mgm /kgm.	DOSE OF GAMMEXANE CON- TAINED IN MIXED ISOMERS mgm /kgm.	DOSE OF PURE GAMMEXANE mgm /kgm.	MORTALITY
98	12.5	6	6/9
—	—	6	24/24

the delta compound was not peripheral since nerve-muscle preparations in intact, anesthetized rabbits showed the same threshold to electrical stimulation before and after suitable doses of this isomer. The beta compound was not studied in this respect. This suggests a direct central depression by the delta and probably also the beta isomer.

d. *Antagonism between the mixed isomers and gammexane.* Using the mixture of isomers previously described it can be noted that an intravenous dose of the mixed isomers prophylactically² given reduces the toxicity of gammexane. The results of these experiments are shown in Table III (cf. Table II for toxicity of the gammexane).

² "Prophylactic administration" of the delta isomer or the isomer mixture refers to the administration of such compounds 10 to 15 minutes prior to the administration of gammexane.

Antagonism between the delta and gamma isomers of 666. Following the prophylactic intravenous administration of the delta isomer, the toxicity of the gammexane is reduced as indicated in Table IV.

e. *The central and cardiovascular actions of gammexane in dogs.* The previously mentioned central and cardiovascular effects of gammexane were further investigated in dogs. In orienting tests using atropinized animals (to prevent early cardiac arrest, vide supra) four animals survived an intravenous dose of 2 mgm./kgm. of gammexane and one of two animals died after the intravenous administration of 4 mgm./kgm. The symptoms observed in all animals were qualitatively the same. Within thirty seconds after injection blepharospasm, generalized tremor and ataxia developed. Marked clonic convulsions rapidly followed. The convulsions persisted approximately 30 seconds followed by an equal period of prostration. This cycle may be repeated more than ten times. Urination, defecation and increased pilomotor activity also frequently occurred.

In the two dogs receiving 4 mgm./kgm. of gammexane death occurred in one after 25 minutes, but recovery was well advanced in the other in two hours.

TABLE IV

Antagonism between the delta and gamma isomers of "666" in rabbits

PROPHYLACTIC DOSE OF DELTA ISOMER	DOSE OF GAMMEXANE	MORTALITY
mgm./kgm.	mgm./kgm.	
50	12	0/1
50	24	6/9
—	6	24/24

The electroencephalographic, electrocardiographic and blood pressure changes were then studied in curarized dogs under local anesthesia and artificial respiration. Blood pressure was recorded from the carotid or femoral artery by direct puncture. The EEG changes were recorded by the Grass Electroencephalograph. Within 30 seconds after the intravenous administration of 4 mgm./kgm. of gammexane the electroencephalogram showed the marked increase in potential and frequency of brain waves characteristic of "grand mal" epilepsy. Concomitantly the blood pressure rose well above 200 mm. of mercury despite the fact that a marked bradycardia developed simultaneously. After 30 seconds the "convulsive episode" in the brain terminated abruptly. Immediately the blood pressure began to fall and the heart began to re-establish a normal rate. The blood pressure did not reach the pre-convulsive level before the onset of another episode. This cycle was sometimes repeated more than 10 times. When such animals were previously atropinized, the central effects and blood pressure responses were unchanged although bradycardia did not occur.

When these experiments were repeated on pentobarbitalized dogs without artificial respiration a different picture was noted. Following the intravenous administration of 4 mgm./kgm. of gammexane no "convulsive pattern" appeared

in the electroencephalogram. Moreover, there was a profound blood pressure fall to levels as low as 30 mm. Hg with a marked bradycardia. These effects gradually subsided and the animals regained the original cardiovascular status within 15 minutes. If such animals were previously atropinized, a blood pressure fall occurred in the absence of bradycardia. Repeated doses of 10 cc./kgm. of control emulsion produced transitory rises of 5 to 10 mm. Hg in blood pressure.

The antagonism between the delta and gamma isomers was also demonstrable when the central actions were observed electroencephalographically in curarized dogs. After pre-treatment with adequate doses of the delta isomer (10-30 mgm./kgm.), the blood pressure fell to levels as low as 50 mm. Hg and gammexane did not produce the electroencephalographic "grand mal" episode. Moreover, the cardiovascular responses of gammexane were also antagonized. The blood pressure fall produced by the delta isomer is a minor factor in this general antagonism since unprotected dogs exhibit typical central and cardiovascular effects despite low blood pressure levels. It should be noted that the delta isomer produced central depression when administered to normal dogs.

On the supposition that the pressor effects of gammexane (in the curarized dog) were produced by sympathetic over-discharge following central stimulation, the effects of sympatholytic drugs were investigated. Yohimbine hydrochloride was used in two experiments. In the first, a curarized dog was given 5 mgm./kgm. of yohimbine hydrochloride. This dose lowered the blood pressure from a level of 130-140 to 80 mm. Hg remaining at this level for the duration of the experiment. A subsequent dose of 4 mgm./kgm. of gammexane did not influence the blood pressure despite the production of numerous "convulsive episodes" in the brain as recorded by the electroencephalograph. In the second experiment, the curarized animal was given 1 mgm./kgm. of yohimbine hydrochloride and 2 mgm./kgm. of atropine; this procedure lowered the animal's blood pressure to 85 mm. Hg. A subsequent 4 mgm./kgm. dose of gammexane produced convulsive brain patterns with concomitant blood pressure rises to 110 mm. of Hg.

In another group of experiments dibenzylbetachloroethylamine hydrochloride (dibenamine) was used as the sympatholytic agent. This drug alone produced variable blood pressure effects. In one animal it caused a marked, transitory rise in blood pressure; in others it caused a sharp, transitory fall in blood pressure. After the initial effects subsided, the blood pressure was usually stabilized at a level 20 to 30 mm. below the control level. Following this procedure a 4 mgm./kgm. dose of gammexane produced the "convulsive brain patterns" and a rise in blood pressure. In some experiments this rise was not as great as that expected in an animal receiving no sympatholytic drug but it was always greater than the blood pressure rise produced by gammexane in yohimbinized dogs.

DISCUSSION. The spatial isomers of hexachlorocyclohexane bring special interest to the study of the relationship between chemical structure and pharmacological activity. Physical differences among stereo-isomers are common and there are many examples of competition between chemically similar com-

pounds for common cell receptors (3, 4). It has been stated by Kuhn (5) that sex of certain algae was determined by the ratio between the amounts of *cis* and *trans* dimethylcrocin present as the sex hormone. Certain derivatives of barbituric acid exhibit a convulsant instead of the usual hypnotic action (6, 7); in these cases either new chemical groups have been introduced or the isomerism is structural rather than spatial. However, direct pharmacological antagonism between stereo-isomers is rare. Concerning the stereo-isomers of hexachlorocyclohexane, physical differences appear in melting points, solubilities, etc. Competition between gammexane and structurally similar inositol has been demonstrated on yeast which require an exogenous source of the alcohol (8). We have been unable to parallel this observation in rabbits (9). Pharmacologically, there is a direct antagonism between gammexane and the other isomers studied (*beta* and *delta*). Gammexane possesses high toxicity and produced marked central stimulation. The *beta* and *delta* compounds are of much lower toxicity and their action on the central nervous system is one of depression. Moreover, the toxicity of gammexane is reduced by prophylactic administration of the *delta* compound. This antagonism is also evident when electroencephalographic and circulatory effects are observed, i.e., not only does the *delta* isomer block the central manifestations, but it also inhibits the cardiovascular effects of gammexane. Such pharmacological contrast in compounds chemically identical except for spatial arrangement of identical substituents appear to be unique.

The few data concerning the alteration of cardiovascular effects of gammexane following yohimbine, dibenamine and atropine suggests that the hypertension and the bradycardia produced by the compound are manifestations of central stimulation carried over to the autonomic nervous system. The hypotension produced by gammexane in pentobarbitalized animals is unexplained and requires further study.

CONCLUSIONS

- 1) The *beta*, *gamma* and *delta* isomers of hexachlorocyclohexane exhibit different toxicities when administered intravenously to rabbits.
- 2) The outstanding pharmacological action of the gammexane in rabbits and dogs is stimulation of the central nervous system.
- 3) The *beta* and *delta* isomers of hexachlorocyclohexane depress the central nervous system of rabbits.
- 4) The *beta* and *delta* isomers antagonize the central effects of gammexane in rabbits and dogs.
- 5) Gammexane produces a "grand mal" type of electroencephalogram, bradycardia and a blood pressure rise in curarized dogs. Atropine blocks the bradycardia but not the pressor effect in such animals.
- 6) In pentobarbitalized dogs, gammexane does not produce changes in the electroencephalographic pattern, but causes bradycardia and a fall in blood pressure. Atropine prevents the bradycardia but does not prevent the blood pressure fall.

7) Yohimbine hydrochloride inhibits the blood pressure rise produced by gammexane without blocking the central effects. Dibenzyl chloroethyl amine hydrochloride (dibenamine) was less effective in this respect.

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THE TREATMENT OF ACUTE POISONING PRODUCED BY GAMMA HEXACHLOROCYCLOHEXANE

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I. INTRODUCTION. The high potency of gammexane (the gamma isomer of hexachlorocyclohexane) as an insecticide has been reported by Slade (1). The oral, subcutaneous and percutaneous toxicity, symptomatology and pathology produced in animals by this compound has been reported by Cameron and Burgess (2). Pharmacological studies by the present authors (3) have shown that gammexane is a powerful stimulant of the central nervous system. The high toxicity and the anticipated widespread use of this compound as an insecticide presents a possible poisoning hazard. Investigations of treatment methods for such poisoning are presented below.

II. EXPERIMENTAL. *The effect of pentobarbital on the poisoning produced by the intravenous administration of gammexane in rabbits.* One volume of a 10% solution of gammexane in peanut oil was emulsified with nine volumes of a 1% soy bean lecithin solution in saline. Following the intravenous administration of the emulsion to rabbits symptoms were observed within two minutes. The animals became restless and appeared apprehensive. Respiration was increased in depth and frequency and blepharospasm appeared. Generalized muscle tremors developed and strong convulsions rapidly ensued. During the convulsions respiration ceased and the heart rate fell to low levels; cardiac arrest often resulted. Examination of the heart at such a time showed complete systole of the left ventricle with auricular diastole. In many cases after cardiac and respiratory failure, it was possible to resuscitate the rabbits by artificial respiration and cardiac massage by gentle intermittent pressure on the thorax. Animals which survived this initial episode exhibited intermittent convulsions between which they remained prostrate. Tremors disappeared but hyperpnea persisted. If death did not occur within one hour, complete recovery usually followed. A dose of 10 cc./kgm. of a control emulsion killed one rabbit in a group of 10. Death apparently resulted from lung edema due to oil embolism.

The intravenous toxicity of gammexane in rabbits is shown in Table I.

The prophylactic, intravenous administration of 20 mgm./kgm. of pentobarbital markedly reduced the mortality produced by intravenously administered gammexane (Table II).

The effect of inositol on the poisoning produced by the intravenous administration of gammexane in rabbits. It has been presumed by Slade (1) that gammexane has the same spatial configuration as *D*-inositol and that the insecticidal action of the former compound might be due to its interference with the inositol metabolism essential for the insect. Kirkwood and Phillips (4) found that the growth of the Gebrüder—Mayer strain of *Saccharomyces cerevisiae*, an inositol requiring yeast,

was inhibited by gammexane. The inhibition produced by other isomers of hexachlorocyclohexane (alpha and beta) was comparatively slight while that produced by the delta isomer was pronounced. In all three cases the inhibition was not affected by the addition of inositol. The gamma isomer (gammexane) markedly inhibited the growth of the yeast and this inhibition was progressively but not completely reversed by exogenous i-inositol. A strain of yeast which did not require an exogenous source of inositol was only slightly inhibited by gammexane.

On the basis of these observations, massive doses of i-inositol were administered prophylactically to rabbits over prolonged periods prior to the administration of lethal doses of gammexane. This procedure did not significantly lower the mortality produced by gammexane (Table III). An apparent antidotal effect was

TABLE I
The intravenous toxicity of gammexane in rabbits

DOSE OF GAMMEXANE	MORTALITY
<i>mgm./kgm.</i>	
4.0	1/7
4.5	9/11
5.0	4/5
6.0	24/24

TABLE II
Pentobarbital treatment of poisoning by intravenous gammexane in rabbits

DOSE OF PENTOBARBITAL	DOSE OF GAMMEXANE	MORTALITY
<i>mgm./kgm.</i>	<i>mgm./kgm.</i>	
20	6	0/1
20	10	0/4
20	18	0/4
20	60	0/6

exhibited by inositol only in one group of rabbits (Experiment 3, Table III). In this experiment gammexane control animals showed unusually long survival periods, in one case as long as 12 hours. Moreover, in another control experiment on this colony of rabbits with the same emulsion, 3 of 11 rabbits survived the gammexane poisoning when glucose was substituted for inositol.

It was then considered that gammexane might exert its action by interfering with glucose metabolism in some manner. However, in repeated experiments on other rabbit colonies with other gammexane emulsions no protection could be demonstrated by glucose, glucose and insulin, or insulin. Thus, protection for inositol and glucose could be demonstrated only against one particular gammexane emulsion and only in one particular group of rabbits. No explanation can be offered for this possible protection in otherwise consistently unsuccessful attempts. If all the inositol experiments are accepted without reservation, it

remains that 4 of 22 rabbits so protected survived poisoning by gammexane. Such protection is obviously inferior to that afforded by pentobarbital.

The results of this study are shown in Table III.

The effect of other isomers of hexachlorocyclohexane on the toxicity of gammexane in rabbits. The beta and delta isomers of hexachlorocyclohexane produce a depression of the central nervous system of animals. These isomers antagonize the central stimulation produced by gammexane and decrease the mortality produced by the latter isomer (3). However, the antidotal value of the alpha, beta, and delta isomers against poisoning produced by gammexane is not as great as that of pentobarbital.

TABLE III

The effect of inositol on the intravenous toxicity of 6 mgm /kgm gammexane in rabbits

EXPERIMENT NO	INOSITOL PROPHYLACTIC TREATMENT			MORTALITY	TIME OF DEATH
	Dose	Route	Time administered prior to gammexane		
	gram/kgm				minutes
1	1.0	i v	1½ hours	4/4	1, 1, 2, 83
	1.0	s c	1½ hours		
	1.0	i v	1 minute		
2	1.0	i v	5 hours	4/4	1, 4, 12, 28
	1 0	s c	5 hours		
	1 0	s c	3½ hours		
	1 0	s c	1 hour		
3	1.0	i v.	2 hours	5/9	2, 3, 5, 60, 95
	1 0	s c	2 hours		
	1 0	i v	10 minutes		
4	1 0	i v	2½ hours	5/5	10, 17, 18, 22, 23
	1 0	s c	2½ hours		
	1 0	i v	10 minutes		

The effect of barbiturates on poisoning produced by the oral administration of gammexane in rabbits. A 10% solution of a highly purified sample of gammexane (Hooker Electrochemical Company D-4050) was prepared in peanut oil.¹ Following the oral administration of this solution symptoms appeared in 30 minutes to 4 hours. Qualitatively the symptoms were the same as those described for the intravenous toxicity of gammexane in rabbits. The oral toxicity of gammexane in rabbits is shown in Table IV.

In the treatment experiments, 200 mgm /kgm of gammexane was administered orally to all animals. The therapeutic agent was always withheld until the onset of symptoms and was administered symptomatically thereafter. In a group of 5 rabbits thus treated, the animals were maintained in a state of slight depression

¹ We are indebted to Dr J S Sconce of Hooker Electrochemical Co, Niagara Falls, for the samples of pure isomers used in the study

for a period of 5 hours with pentobarbital, and treatment was discontinued thereafter. All such animals subsequently died. In two other groups of rabbits, therapy was continued for a period of 32 hours. Eleven rabbits were treated with pentobarbital and 15 were treated with phenobarbital. During the period of therapy, three rabbits died, one in the pentobarbital group and two in the phenobarbital group. However, in each case, the animal died before it was possible to interrupt the convulsion by treatment.

After treatment was discontinued, 5 additional rabbits of the phenobarbital group and 8 of the pentobarbital group died. Some deaths occurred as late as 5 days and, in such animals, convulsions were the terminal episode. The results appear in Table V.

TABLE IV
The oral toxicity of gammexane in rabbits

DOSE OF GAMMEXANE	MORTALITY
mgm./kgm.	
50	2/8
100	10/10
200	10/10

TABLE V
The effect of barbiturates against poisoning by oral gammexane in rabbits

NO. OF ANIMALS	DOSE OF GAMMEXANE	BARBITURATE*	DURATION OF TREATMENT	DEATHS DURING TREATMENT	DEATHS AFTER TREATMENT WAS DISCONTINUED
	mgm./kgm.		hours		
5	200	Pentobarbital	5	none	5
11	200	Pentobarbital	32	1	8
15	200	Phenobarbital	32	2	5

* Administered symptomatically.

It may be mentioned that pentobarbital exhibited a rapid but short-lasting effect. This made frequent treatment necessary. Phenobarbital exerted lasting protection in doses causing less depression than pentobarbital. The former animals were alert and moved freely about, whereas those treated with pentobarbital went into convulsions unless kept deeply depressed.

The effect of barbiturates on poisoning produced by oral administration of gammexane in dogs. The initial symptoms produced by the oral administration of gammexane in dogs appear in from 30 minutes to 4 hours and the toxic syndrome is similar to that produced in the rabbit. The oral toxicity of this compound in dogs is depicted in Table VI.

Treatment of gammexane poisoned dogs is limited to eight animals. All were given 200 mgm./kgm. of gammexane by stomach tube. Six were treated with pentobarbital and two with phenobarbital.

The treatment agents were administered symptomatically and usually by the intravenous route. Pentobarbital was given in increments of 5-20 mgm./kgm. and phenobarbital in increments of 25 mgm./kgm.

In the pentobarbital group the total dosage ranged from 30-65 mgm./kgm. during the first day. One animal died during the first 18 hours and the others were convulsive after 24 hours. A dose of 10-15 mgm./kgm. of pentobarbital at this time prevented further convulsions in 4 of the 5 remaining animals. These 4 animals exhibited a mild hyperexcitability and occasional muscular twitching for 4 or 5 days; thereafter they appeared completely normal. No subsequent signs of residual abnormality developed. The 5th dog became convulsive on the 4th day and was given 10 mgm./kgm. of pentobarbital; symptoms were promptly and completely controlled. However, this animal was found dead on the following day.

TABLE VI
The oral toxicity of gammexane in dogs

DOSE OF GAMMEXANE mgm /kgm.	MORTALITY
300	1/1
200	1/1
100	3/3
50	4/7

TABLE VII
Barbiturate treatment of oral gammexane poisoning in dogs

NUMBER OF DOGS	DOSE OF GAMMEXANE mgm /kgm	BARBITURATE	DEATHS
6	200	Pentobarbital	2
2	200	Phenobarbital	1

The first dog treated with phenobarbital received 125 mgm./kgm. intravenously during a 2½ hour period. Despite this massive dose of the depressant, mild intermittent convulsive seizures occurred. This dog remained deeply depressed throughout the 13 day survival period. Throughout this time it was necessary to sustain the animal constantly by infusion of glucose-saline solution since it could not eat and dehydration was severe. Despite the marked depression gross twitching of the face and neck muscles persisted until death.

The second phenobarbital-treated dog received 75 mgm./kgm. of the treatment agent during a period of 2½ hours. On the following day, this animal was drowsy but otherwise normal and on the third day, it was normal in all respects. No signs of residual damage have since been observed. The results of this study are shown in Table VII.

COMMENT. Pentobarbital or phenobarbital effectively antagonize the toxic effects of large intravenous doses of gammexane. This is true also of large oral

doses, but in this case, symptomatic treatment is necessary. In the limited number of experiments on dogs it appeared that recovery was better when a minimum of narcosis was employed and the symptoms of central stimulation were not completely abolished; this was most evident in the case of phenobarbital. In the dog in which deep narcosis was produced by phenobarbital, the depression persisted for a period of days until the animal finally died. Hence, it is the belief of the authors that the ideal therapeutic regime would be the administration of a small dose of phenobarbital with subsequent, symptomatic doses of a short-acting barbiturate to control violent convulsive seizures. The value of artificial respiration and cardiac massage, as previously described, is re-emphasized.

The persistence of the effects of gammexane for several days following large oral doses deserves comment inasmuch as the effects of the intravenous toxic equivalent (in terms of the LD_{50}) persist for only a matter of hours. The reason for such long persistence has not been determined, but one possibility is hepatic storage of gammexane with subsequent slow release for excretion and/or detoxification. However, it is clear that treatment by an appropriate depressant under such circumstances is essential and that adequate symptomatic control appears to prevent functional injury by high concentrations of gammexane in body fluids for extended periods of time.

The absence of permanent functional injury in recovered animals is noteworthy. Animals surviving all experiments were observed over prolonged periods. These animals showed no functional signs of residual damage. Serum bilirubin and non-protein nitrogen determinations in the dogs remained normal throughout. Autopsy findings on early sacrificed dogs and rabbits showed some changes but these changes were not present in animals sacrificed at later periods.

The antagonism between the isomers of hexachlorocyclohexane is of pharmacological interest but appears of very little value in the treatment of poisoning produced by gammexane. I-inositol shows no promise for treatment of such poisoning in mammals.

CONCLUSIONS

Pentobarbital and phenobarbital effectively protect against acute, oral and intravenous poisoning by gammexane in dogs and rabbits.

The delta and probably the beta isomers of hexachlorocyclohexane antagonize the central effects of gammexane, but the former isomers are inferior to pentobarbital or phenobarbital in the treatment of poisoning produced by gammexane.

I-inositol exhibited little protection against poisoning produced by the intravenous administration of gammexane in rabbits.

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THE EFFECT OF CALCIUM ON THE CARDIOVASCULAR STIMULATION PRODUCED BY ACETYLCHOLINE

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INTRODUCTION. It has long been recognized that acetylcholine produces a blood pressure rise in atropinized animals in many respects similar to the well-known effect of epinephrine (1-13). This effect is more easily demonstrable if the animal is treated with an anticholinesterase compound. The response to acetylcholine has been attributed to stimulation of sympathetic ganglia (9). Recent investigations by McDowall (14) and Hoffmann (15) have shown that this compound also has a stimulating action on the isolated, atropinized heart, i.e. rate and force of contraction are increased. McDowall stated that the action persisted after the administration of sufficient nicotine to paralyze autonomic ganglia. He concluded that acetylcholine stimulated heart muscle directly. Hoffmann reported that nicotine abolished the stimulating action of acetylcholine and in addition demonstrated that the perfusate from the isolated heart contained an epinephrine-like substance following the administration of acetylcholine. He assumed that the ester acted on nervous structures in the heart equivalent to sympathetic ganglion or chromaffine tissue.

The results presented in the following report indicate that calcium exerts an important conditioning influence in these cardiovascular responses. Several ganglionic and sympathetic neuro-effector blocking agents were used with the view to gaining further insight into the "nicotinic" actions of acetylcholine upon ganglia, especially with respect to the rôle of calcium.

EXPERIMENTAL. I. *The pressor response to acetylcholine.* Cats and dogs were anesthetized with sodium pentobarbital (30 mgm./kgm., intravenously) and atropinized (1-5 mgm./kgm., intravenously). A suitable intravenous dose of an anticholinesterase compound (usually 0.1 mgm./kgm. of DFP) was also administered. Blood pressure measurements were recorded from the carotid artery with a mercury manometer. Artificial respiration was employed when necessary. The experiments were essentially as follows: Reproducible, sub-maximal pressor responses to intravenously administered acetylcholine (50-300 gamma/kgm.) were obtained (fig. I, A). These responses were then blocked in a given experiment, by nicotine, dimethyl piperidine (16), magnesium sulfate or intocostin (fig. I, B). The pressor response of acetylcholine was then in turn restored by the intravenous administration of calcium chloride (fig. I, C).

The ganglionic blocking effect of nicotine is well known. It was achieved in this study by injecting the drug intravenously in increments of 0.1 or 0.2 mgm./kgm. until the pressor effect of acetylcholine was abolished; this usually required total doses of 0.5 to 1.0 mgm./kgm. of nicotine. A similar result was obtained with magnesium sulfate after slow intravenous injection of divided doses of the

salt. It was sometimes necessary to administer as much as 400 mgm./kgm. in a 30 minute period before this effect could be demonstrated. Curare was given intravenously in increments of 5-10 units/kgm. (The provisional unit of intocostin is equivalent to the potency of 0.15 mgm. of d-tubocurarine chloride.) until block occurred; this usually required 20-40 units/kgm. of intocostin over a period of 30 to 60 minutes. Dimethylpiperidine was given intravenously in 10 to 20 mgm./kgm. increments at 5 minute intervals until block resulted; total dose of 50 mgm./kgm. was usually sufficient to produce this effect.

A single intravenous dose of 50 mgm./kgm. of CaCl_2 was usually sufficient to restore the pressor effect of acetylcholine.

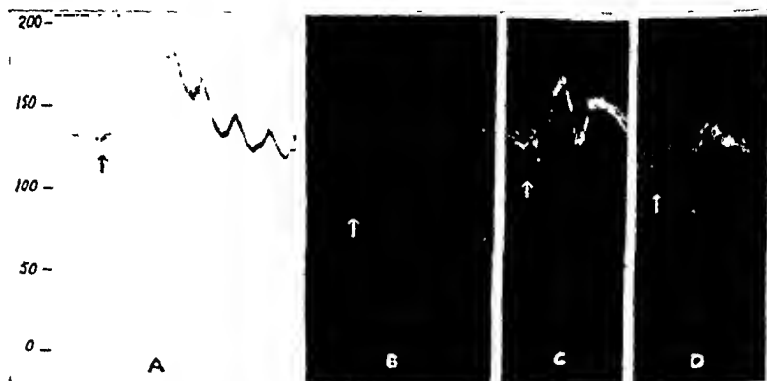


FIG. 1. BLOOD PRESSURE RESPONSES PRODUCED BY ACETYLCHOLINE IN THE CAT

The animal was previously anesthetized with pentobarbital and treated with 3 mgm./kgm. of atropine sulfate and 100 gamma/kgm. of DFP. Arrows indicate the injection of 200 gamma/kgm. of acetylcholine. The base line indicates zero pressure. All drugs were administered intravenously throughout the experiment.

A. Pressor response to acetylcholine.

B. Block of pressor response to acetylcholine after the administration (during a 45 minute period) of 400 mgm./kgm. of $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$.

C. Restoration of pressor response of acetylcholine after the administration (during 30 minutes) of 75 mgm./kgm. of CaCl_2 .

D. Re-block of pressor response of acetylcholine after the administration of 100 mgm./kgm. of $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$.

II. The effect of calcium on depressed sympathetic ganglia (superior cervical).

In an attempt to demonstrate parallelism between the alterations in the pressor effect to acetylcholine and responses to electrical stimulation of sympathetic ganglia, several experiments were carried out as follows: The vago-sympathetic trunk of pentobarbitalized dogs and the sympathetic trunk of cats was exposed in the neck, and shielded platinum electrodes were applied. The threshold voltage necessary to produce the typical ocular signs upon preganglionic stimulation was then determined. The source of stimulus was a Grass Model 3 Stimulator. The depressant (nicotine, magnesium sulfate, dimethylpiperidine or intocostin) was then administered intravenously in divided doses until a significant increase in the ocular threshold was observed. Calcium chloride was

then given intravenously in divided doses of 50 mgm./kgm. and the ocular threshold was again determined between doses. By this technique, it was found that each of the above depressant drugs increased the threshold. Moreover, calcium ion further increased the voltage necessary to produce ocular responses.

III. *The stimulating action of acetylcholine on the isolated rabbit heart.* The possibility that the heart might be involved to a considerable extent in the production of the pressor response to acetylcholine led to the following experiments.

The coronary arteries of isolated rabbit hearts were perfused under pressure by the Langendorff method. A modified Locke solution (17), warmed to 38°C. and saturated with 5% CO₂ and 95% oxygen, was used. Atropine (0.5 mgm./liter) was also added to this perfusion fluid. The drugs to be studied were introduced by one of two methods. In some cases doses were injected into the tubing close to the heart. In other instances more dilute solutions of the drugs were infused via a small bore needle into the tubing just above the aortic cannula by means of a constant-flow mercury pump. In all experiments an initial dose of 50 gamma of atropine and 10 gamma of DFP was administered. Mechanograms were recorded by means of a suitable isotonic lever attached to the apex.

Wide individual variations were noted between heart preparations, each of which presented an individual problem of dosage. Doses of the depressants which were inadequate for some hearts irreversibly poisoned others and many experiments failed. However, the combination of the individual dosage and infusion techniques provided a practical solution in many experiments. Best results were often obtained in experiments of short duration when block was produced within 20 minutes and reversal was then accomplished in a like period.

(a) The potentiating effect of CaCl₂ on the cardiac stimulation produced by acetylcholine and epinephrine. Acetylcholine and epinephrine were always administered by the individual dose method. Doses of 50–250 gamma of acetylcholine and 0.1–1.0 gamma of epinephrine caused quantitatively reproducible increases in amplitude of contraction. When calcium chloride was introduced by infusion at the rate of 0.6 to 0.9 mg. per minute, previously submaximal stimulating doses of acetylcholine or epinephrine produced greater responses; however, subthreshold doses were not rendered effective.

(b) Calcium chloride reversal of cardiac effects of "ganglionic blocking" agents. The general procedure in these experiments is described below using dimethylpiperidine as the blocking agent. After the determination of effective submaximal doses of acetylcholine (fig. II, 1, 2), the "blocking agent" (dimethylpiperidine) was infused at a constant rate of 30 gamma/minute. This rate was often sufficient to abolish or to significantly decrease (within 5 minutes) the stimulation produced by the predetermined doses of acetylcholine (fig. II, 3). In heart preparations in which this infusion rate was inadequate it was sometimes possible to maintain a block by perfusion after one or more priming doses of dimethylpiperidine (5–10 mg.). After a constant block was established (fig. II, 3) calcium chloride was infused at the rate of 0.6–0.9 mgm./minute without altering the infusion rate of dimethylpiperidine. During this procedure the

stimulating effect of acetylcholine could again be demonstrated (fig. II; 4, 5). Interruption of calcium chloride infusion reestablished block toward acetylcholine (fig. II, 6, 7). However, after the cessation of calcium chloride infusion, longer periods were necessary before dimethylpiperidine restored a block. These reversals of actions could be repeated as long as the heart preparation remained active.

Cocaine (25-30 gamma/minute), nicotine (3-5 gamma/minute), magnesium sulfate (7.5-15 mgm./minute), intocostin (0.025-0.05 units/minute) produced similar results by the procedure described above for dimethylpiperidine.

IV. *The production of a sympathomimetic substance by the isolated heart following cardiac stimulation by acetylcholine and epinephrine.* Preliminary experiments demonstrated the formation of a sympathomimetic substance in the isolated heart following the administration of acetylcholine and possibly epinephrine.



FIG. II. THE EFFECT OF ACETYLCHOLINE ON THE ISOLATED HEART

The heart was prepared as described in the text. Drum stopped at points indicated by X.

1. 75 gamma of acetylcholine.
2. 100 gamma of acetylcholine.
3. 100 gamma of acetylcholine four minutes after the start of a constant infusion of dimethylpiperidine.
4. 75 gamma of acetylcholine during constant infusion of calcium chloride.
5. 100 gamma of acetylcholine ten minutes after the start of the calcium infusion. No interruption to dimethylpiperidine infusion.
6. 75 gamma of acetylcholine 28 minutes after interruption of the calcium infusion. No interruption to dimethylpiperidine infusion.
7. 100 gamma of acetylcholine 30 minutes after interruption of the calcium infusion. Dimethylpiperidine infusion was running.

Isolated rabbit intestine was used as the test object in these experiments and myograms were recorded isotonicly. The tissue bath medium employed was the same as that used in the experiments on the isolated hearts (including 0.5 mgm./liter of atropine sulfate).

Doses of 0.1 gamma to 0.5 gamma of epinephrine applied directly to the intestine in a 40 cc. tissue bath, produced a definite reduction of the intestinal tone and rhythmicity (fig. III, 1; Fig. IV, 1). Ten cc. portions of perfusate, collected from an isolated heart after stimulation by 0.1 gamma to 0.5 gamma doses of epinephrine, also relaxed the intestine when added to 30 cc. of tissue bath medium. This relaxation was of lesser magnitude than that produced by direct addition of epinephrine to the bath medium (fig. III, 4; fig. IV, 2). Ten cc. portions of perfusate from an unstimulated heart produced no intestinal effects when added to 30 cc. of tissue bath medium (fig. IV, 4). When the heart was then perfused with CaCl_2 (0.9 mgm./minute) the resulting effluante still

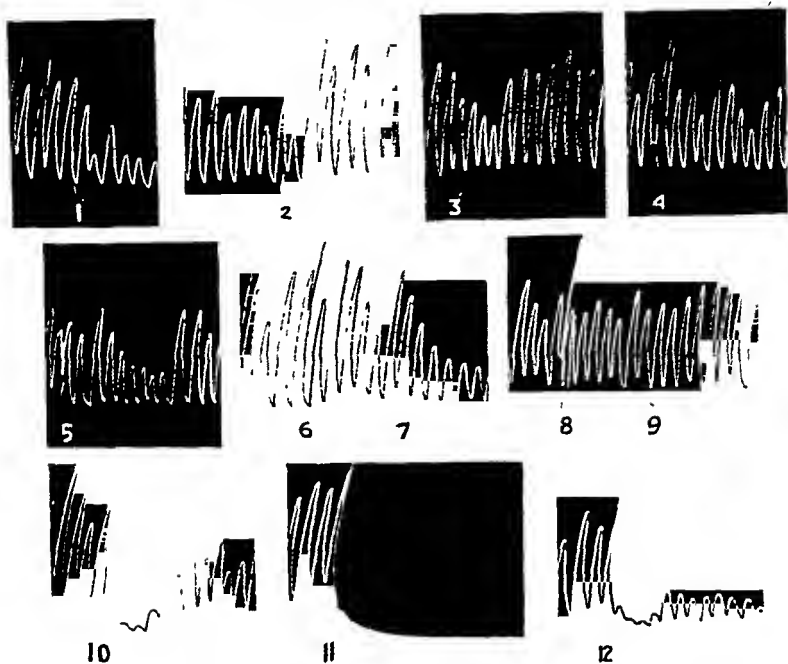


FIG. III. THE RELEASE OF A SYMPATHOMIMETIC SUBSTANCE IN THE ISOLATED HEART AS DEMONSTRATED ON THE ISOLATED INTESTINE

The isolated tissues were prepared as described in the text. Myograms represent tone and motility of the intestinal segment. The numerals indicate the addition of the test substance to the tissue medium containing the intestine.

1. 0.4 gamma of epinephrine added to 40 cc. of intestinal bath medium.
2. 150 gamma of acetylcholine added to 40 cc. of the intestinal bath medium.
3. 100 gamma of acetylcholine added to 40 cc. of the intestinal bath medium.
4. 10 cc. of heart perfusate collected during cardiac stimulation by 0.4 gamma of epinephrine, added to 30 cc. of intestinal bath medium.
5. 10 cc. of heart perfusate collected during cardiac stimulation by 150 gamma of acetylcholine, added to 30 cc. of intestinal bath medium.
6. 10 cc. of heart perfusate collected during the cardiac infusion of CaCl_2 (0.9 mgm./minute), added to 30 cc. of the intestinal bath medium.
7. 0.4 gamma of epinephrine added to the intestinal bath medium in the presence of the heart perfusate described in 6.
8. 10 cc. of heart perfusate collected during cardiac infusion of CaCl_2 (0.9 mgm./minute), added to 30 cc. of the intestinal bath medium.
9. 100 gamma of acetylcholine added to the intestinal bath medium in the presence of the heart perfusate described in 8.
10. 10 cc. of perfusate collected from a CaCl_2 infused heart during cardiac stimulation by 0.4 gamma of epinephrine, added to the intestinal bath medium.
11. 10 cc. of perfusate from a calcium infused heart collected during cardiac stimulation by 150 gamma of acetylcholine, added to 30 cc. of intestinal bath medium.
12. 10 cc. of perfusate from a calcium infused heart collected during cardiac stimulation by 100 gamma of acetylcholine, added to 30 cc. of intestinal bath medium.

exhibited no intestinal activity (fig. III, 6, 8). Moreover, previously established submaximal doses of epinephrine applied directly to the intestines in the presence of such a perfusate did not increase the inhibitory properties (fig. III, 7); however, if a similar dose of epinephrine was injected into the isolated heart during

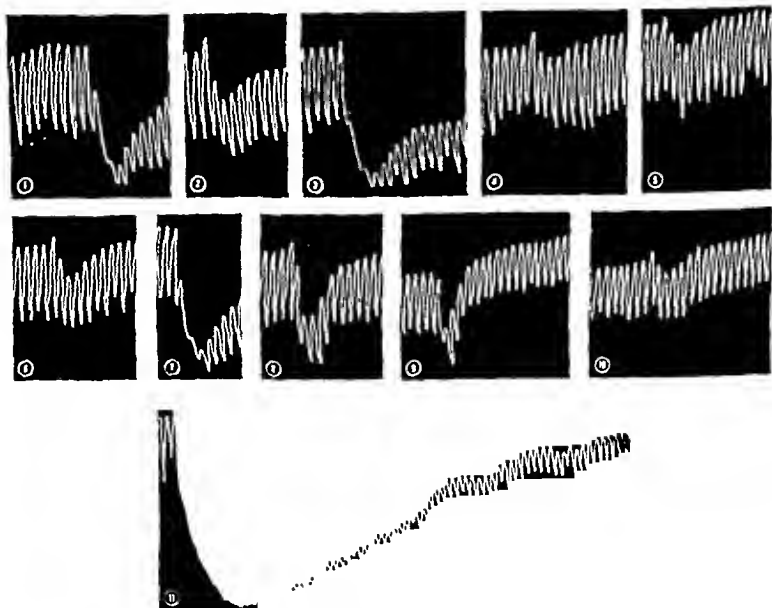


FIG. IV. THE RELEASE OF A SYMPATHOMIMETIC SUBSTANCE IN THE ISOLATED HEART AS DEMONSTRATED ON THE ISOLATED INTESTINE

The isolated tissues were prepared as described in the text. Myograms represent tone and motility of intestinal segment. The numerals indicate the addition of test substance to tissue bath medium containing the intestine.

1. 0.4 gamma of epinephrine added to 40 cc. of intestinal bath medium.
2. 10 cc. of heart perfusate collected during cardiac stimulation by 0.4 gamma of epinephrine, added to 30 cc. of intestinal bath medium.
3. 10 cc. of heart perfusate collected during cardiac stimulation by 200 gamma of acetylcholine, added to 30 cc. of intestinal bath medium.
4. 10 cc. of normal heart perfusate added to 30 cc. of intestinal bath medium.
5. 10 cc. of heart perfusate collected during dimethylpiperidine infusion (30 gamma/minute), added to 30 cc. of intestinal bath medium.
6. 10 cc. of perfusate collected after the injection of 200 gamma of acetylcholine from a heart which was being infused with dimethylpiperidine, added to 30 cc. of the intestinal bath medium.
7. 0.4 gamma of epinephrine added to 40 cc. of intestinal bath medium.
8. 10 cc. of perfusate, from a heart under dimethylpiperidine infusion, collected during cardiac stimulation by 0.4 gamma of epinephrine, added to 30 cc. of the intestinal bath medium.
9. 10 cc. of calcium chloride solution (0.23 mgm./cc.) added to 30 cc. of the intestinal bath medium.
10. 10 cc. of perfusate collected after the injection of 200 gamma of acetylcholine from a heart which was being infused with dimethylpiperidine, added to 30 cc. of the intestinal bath medium containing added calcium as described in 9.
11. 10 cc. of perfusate collected after the injection of 200 gamma of acetylcholine from a heart which was being infused with dimethylpiperidine and calcium chloride (0.9 mgm./minute), added to 30 cc. of intestinal bath medium.

calcium infusion, the intestinal inhibitory effect was markedly increased (compare fig. III, 4 and fig. III, 10).

Doses of 100 to 250 gamma of acetylcholine applied directly to the intestine produced either no effect or an increase in intestinal tone (fig. III, 2, 3). In bath fluid containing no atropine the tonal increase was greater. Similar doses

of acetylcholine produced cardiac stimulation in the isolated heart and the perfusate therefrom caused definite decrease in the tonus and rhythmicity of the intestinal strip (fig. III, 5; fig. IV, 3). As previously described, the heart perfusate itself or perfusate collected during cardiac perfusion with CaCl_2 produced no effect on the isolated intestines. Acetylcholine produced no significant direct effect on the intestine even in the presence of perfusate collected during calcium perfusion of the heart (fig. III, 9). However, when a previously tested dose of acetylcholine was injected into the heart during calcium infusion, the intestinal inhibitory effect of the perfusate was markedly increased. (Compare fig. III, 5 and fig. III, 11 and 12).

The ability of dimethylpiperidine to block the cardio-stimulating action of acetylcholine has been described earlier in this report. Figure IV, 3, demonstrates the intestinal inhibition produced by heart perfusate following cardiac stimulation by acetylcholine. When the heart was then perfused with dimethylpiperidine (30 gamma/minute) control tests of the perfusate showed no intestinal effects (fig. IV, 5). Injection of tested doses of epinephrine into the heart during this perfusion caused cardiac stimulation and the effluat produced the inhibitory effect on the intestinal strip, a circumstance similar to that before dimethylpiperidine (compare fig. IV, 2 and fig. IV, 8). However, normally effective doses of acetylcholine injected into the heart at this time produced little cardiac stimulation and the perfusate produced little effect on the isolated intestine (fig. IV, 6). Also, when the same perfusate was added to a tissue bath medium containing added calcium no intestinal effect was produced (fig. IV, 10). However, acetylcholine again produced cardiac stimulation and the perfusate caused definite relaxation of the intestinal strip if calcium (0.9 mgm./minute) was added to the perfusion fluid containing dimethylpiperidine (fig. IV, 11).

DISCUSSION. The vasopressor activity exhibited by acetylcholine under suitable conditions has been considered the result of ganglionic stimulation, and blocking of this effect by nicotine has been ascribed to ganglionic depression. Cardiac stimulation by acetylcholine and blocking of this effect by ganglionic depressants can be demonstrated on the isolated heart. Calcium ion reverses the "nicotinic blocking action" on both blood pressure and the isolated heart. It seems probable, therefore, that the cardiac effects described are important components of the pressor activity observed in the intact animal. The experiments presented herein indicate that calcium exerts a fundamental rôle in these cardiovascular responses. Many workers have studied the cardiovascular effects of the calcium ion, but the mechanisms by which calcium ion brings about its effects are still not fully understood. Complete explanations of the mechanisms involved cannot be advanced at the present time, but some suggestions concerning the mechanism of the cardiac effects appear without reference to the exact cardiac tissue(s) involved. Hoffmann (15) has previously stated that acetylcholine stimulated the isolated heart associated with the liberation of a sympathomimetic substance. Our own experiments confirm these observations. The liberation of this substance is augmented by calcium ion and prevented by "nicotinic" blocking agents (e.g. dimethylpiperidine). Under appropriate conditions calcium ion reverses the effects produced by such depressants. Since

the effect of epinephrine on the isolated heart is also potentiated by calcium ion and since the perfusate from an epinephrine-stimulated heart causes increased effects on intestinal strips, the possibility exists that epinephrine also produces the intracardiac liberation of other sympathomimetic agents. This possibility is being investigated. Possible changes in coronary flow caused by the several substances studied have not been measured, but these are not expected to alter materially the conclusions regarding the liberation of a sympathomimetic substance.

The vasopressor responses produced by acetylcholine under conditions of intact circulation present greater complications. Other investigators have indicated that an epinephrine-like substance is released following the administration of acetylcholine. Koppányi et al. (1) reported that a rise of blood pressure occurred in a recipient dog when transfer of blood was made following acetylcholine administration to a donor animal. They state that the pressor effect of acetylcholine is localized in the sympathetic ganglia. However, the sympathomimetic substance produced in the heart itself would represent a part of the pressor activity observed in such experiments. It is also apparent that in addition to its well-known function in the maintenance of cardiac contractility and excitability, calcium ion exercises a basic rôle in the ganglionic and cardiac effects of the several drugs considered in this report and should prove valuable in further investigation of the pharmacological mechanisms involved.

Since calcium restores the cardiac response to acetylcholine after "nicotine block" and since such reversal could not be demonstrated upon the superior cervical ganglion, it would appear that the rôle of sympathetic ganglia in the vasopressor responses of acetylcholine is of minor importance. It is in agreement with the observations of Bronk and co-workers (18, 19) that high concentrations of calcium block synaptic transmission. This could account for the higher threshold to preganglionic stimulation produced by calcium in our experiments, and may not be a reflection of altered capacity of the ganglion to produce a sympathomimetic agent. It should be re-emphasized that in our experiments only the pre-ganglionic fibers of the superior cervical ganglion were stimulated and the possibility of changes in the threshold of the post-ganglionic neurones and of the effector organs were not eliminated. Moreover, effects on this ganglion may not be representative of those participating in the pressor responses.

The work of DuBois, Albaum and Potter (20), who demonstrated that anesthetic doses of magnesium sulfate inhibit the breakdown of adenosinetriphosphate, has contributed to the understanding of the nature of the action of the magnesium ion upon the central nervous system, and since calcium ion is known to favor adenosine triphosphate breakdown, the well-known calcium-magnesium antagonism upon the central nervous system may be more clearly understood. However, it is not yet clear whether this mechanism is related to the cardiac phenomena described in this report.

CONCLUSIONS

1. Acetylcholine exerts a pressor effect on the blood pressure of atropinized animals and has a "stimulating" effect on the isolated, atropinized heart.

2. Ca potentiates the epinephrine and acetylcholine "stimulating" action on the heart.

3. Acetylcholine and perhaps epinephrine causes the liberation of a sympathomimetic substance in the isolated heart. This effect is potentiated by calcium and prevented by dimethylpiperidine.

4. Nicotine, intocostin, $MgSO_4$ and dimethylpiperidine block the pressor effect of acetylcholine and also the "stimulating" effect on the heart.

5. When the cardio-stimulating effect of acetylcholine is inhibited by dimethylpiperidine, cocains, nicotine, or curare, the epinephrine response remains unaltered.

6. Calcium restores the pressor and heart-stimulating effects of acetylcholine after these actions have been previously blocked by nicotine, $MgSO_4$, intocostin or dimethylpiperidine. Similarly calcium restores the "sympathogenic" effect of acetylcholine after this action has been previously blocked by dimethylpiperidine.

7. Nicotine, $MgSO_4$, intocostin, or dimethylpiperidine increase the preganglionic threshold stimulus for the ocular responses following stimulation of the cervical sympathetic trunk.

8. When the preganglionic threshold stimulus for the ocular responses resulting from stimulation of the sympathetic trunk is increased by nicotine, $MgSO_4$, intocostin, or dimethylpiperidine, subsequent administration of calcium further raises this threshold.

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INHIBITION OF THE CHOLINESTERASE ACTIVITY OF HUMAN BLOOD PLASMA AND ERYTHROCYTE STROMATA BY ALKYLATED PHOSPHORUS COMPOUNDS¹

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Several phosphate esters have been found to be potent inhibitors of certain ester-splitting enzymes. The first observations appear to have been made in 1941 with dimethyl fluorophosphate by Adrian and Feldberg and their collaborators who, in 1942, recognized di-isopropyl fluorophosphate (DFP) to be a potent inhibitor of the cholinesterase activity of various tissues (1). Bloch in 1942 (2), and Hottinger and Bloch in 1943 (3) published a careful study of the anticholinesterase activity of the isomeric tricresyl phosphates. Recently Dubois and Mangum (4) ascribed the toxic properties of hexaethyl tetraphosphate to its inhibitory effect upon acetylcholine hydrolyzing enzymes.

The work to be presented below was undertaken in order to shed further light on the reactions involved in the inactivation of cholinesterase by neutral phosphate esters.³

METHODS: The enzyme preparations used for the studies *in vitro* were purified human plasma esterase (Fractions IV-6 or IV-6-3 of the sebeme of Cohn, et al. (5))⁴ and human erythrocyte stromata prepared by hypotonic hemolysis and washed to remove all but traces of hemoglobin (6). Cholinesterase activities were determined using the manometric method of Ammon (7). The reaction medium was 0.025 *M* sodium bicarbonate buffer in 0.25 *M* NaCl; the substrate concentration for plasma esterase determination was chosen as 0.08 *M* acetylcholine bromide (Eastman Kodak Company, once recrystallized), while 0.008 *M* acetylcholine bromide was employed in the erythrocyte cholinesterase determinations. Results are expressed as millimoles of acetylcholine hydrolyzed per hour by one liter of the solution of plasma esterase or the suspension of erythrocyte stromata. Percentage inhibitions were calculated by comparing the rate of reaction in the presence of an inhibitor with that of the same enzyme preparation in the absence of the inhibitor. The molar concentration of any inhibitor which resulted in 50% inhibition of a 0.01% solution of Fraction IV-6 will be referred to below as I_{50} .

In the comparative studies, the inhibitor solutions were prepared directly from the liquid substances. Since solutions in absolute ethanol of hexaethyl tetraphosphate as well as of

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⁴ The products of plasma fractionation were developed, from blood collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract, recommended by The Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

tetraethyl pyrophosphate are stable over long periods of time and can be pipetted, such solutions were used. In view of the pronounced surface activity and the remarkable potency of most of the compounds studied, it was found necessary to clean all glassware either by steaming in an autoclave at 20 lb. per sq. in. for 20 minutes, or by immersion for 12 hours or longer in a mixture of equal parts of concentrated nitric and sulfuric acids.

Cellophane bags (Visking Sausage Casing Co.) were used in experiments involving dialysis. Two experimental arrangements were employed. In one, enzyme solutions were placed inside the bag, and a smaller volume of enzyme solution containing inhibitor was placed outside in a fairly narrow space between the bag and a suitable test tube. The whole arrangement was stoppered, and agitated every few hours. In another arrangement enzyme solution containing inhibitor (especially material containing P^{32}) was placed inside a bag and dialysis was carried out against a slow stream (2 cc. per min.) of 0.9% NaCl delivered through a continuous drip assembly to a suitably narrow tube holding the bag. The dialyses were carried out at room temperature.

Deproteinization of solutions containing enzyme and inhibitor was carried out by the addition of two volumes of 95% ethanol to one volume of the enzyme solution at pH 7.4 and room temperature. The precipitate was separated by centrifugation.

The relative P^{32} content of samples used in this work was determined by means of an end-window counter. Precipitates, because of the small masses involved, were collected on the flattened bottoms of plastic test tubes, cut away from the bulk of the tubes, and tested directly. Supernatants were digested at pH 10 for 6 hours at 90°C. to assure destruction of the pyrophosphates, and were then evaporated, drop by drop, in aluminum planchets on the steam table.

Unless otherwise specified concentrations of phosphate esters are given in terms of dilutions (10^{-6} is equivalent to 1 gram in 10^6 cc.) rather than of molarities which have very little meaning in view of present ignorance of the molecular weights of these compounds.

RESULTS: Table 1 presents the results of testing a series of compounds for their effectiveness in inhibiting the esterase activity of Fraction IV-6. Of the simple trialkyl phosphates tested, only tricresyl phosphate is potent in this respect. All the polyphosphates, phosphophosphines, thio-, and sulfone phosphates tested were found to be active esterase inhibitors. A fluorophosphate is also listed for comparative purposes. In view of the complex chemistry involved, the structural formulae of many of the compounds cannot, at this time, be accepted as unequivocally established.

Compounds 2, tetraethyl pyrophosphate, and 10, hexaethyl tetrapolyphosphate, were selected for further study, and will be referred to below as TEPP and HETP, respectively.

Figure 1 shows the relation between the concentration of TEPP and the degree of inhibition of Fraction IV-6 and of erythrocyte cholinesterase. As in the case of DFP (8), plasma esterase is much more sensitive to the action of this inhibitor than is the erythrocyte enzyme. A linear relation between inhibitor concentration and the degree of enzyme inhibition holds over most of the range for TEPP and Fraction IV-6, as well as for TEPP and erythrocyte cholinesterase.

In order to establish whether the inactivation of Fraction IV-6 by TEPP can be reversed in any measurable degree by removing the inhibitor, two tests were applied. 1. TEPP loses its inhibitory activity on standing in aqueous solution as a result of hydrolysis to inactive alkylphosphoric acids. However, when the esterase of Fraction IV-6 was partially inactivated by the use of TEPP and the

TABLE 1

The esterase inhibiting activity of various phosphorus compounds

COMPOUND		I ₅₀ ^a
No.	Structure	
(1)		2.0×10^{-4} 8.6×10^{-10} 2.8×10^{-8} 1.2×10^{-4}
(2)	R	
(3)	CH ₃	
(4)	C ₂ H ₅	
(5)		1.7×10^{-8}
(6)		1.6×10^{-9}
(7)		2.1×10^{-9} 4.0×10^{-8}
(8)		
(9)		4.8×10^{-9}
(10)		1.0×10^{-9}
(11)		6.5×10^{-8}

TABLE 1.—Continued

COMPOUND		I_{50}^*
No.	Structure	
(12)	$\begin{array}{c} \text{P}(\text{OC}_2\text{H}_5)_4 \\ \\ \text{P}(\text{OC}_2\text{H}_5)_3 \\ \\ \text{P}(\text{OC}_2\text{H}_5)_2 \end{array}$	4.3×10^{-8}
(13)	$\text{O}=\text{P}(\text{OR})_2$	
(14)	R	$>10^{-4}$
(15)	C_2H_5 $n\text{-C}_8\text{H}_{17}$ $o\text{-C}_6\text{H}_4\text{CH}_3$	$>10^{-4}$ $\sim 10^{-7}$
(16)	$\text{O}=\text{P} \begin{array}{c} \text{F} \\ \diagup \\ \text{O}=\text{P} \end{array} \left[\begin{array}{c} \text{CH}_2 \\ \diagup \text{O}-\text{CH} \\ \diagdown \text{CH}_2 \end{array} \right]_2$	5.0×10^{-9}

* I_{50} = The concentration in Mol/L of inhibitor required to produce 50% inhibition of a 0.01% solution of Fraction IV-6.

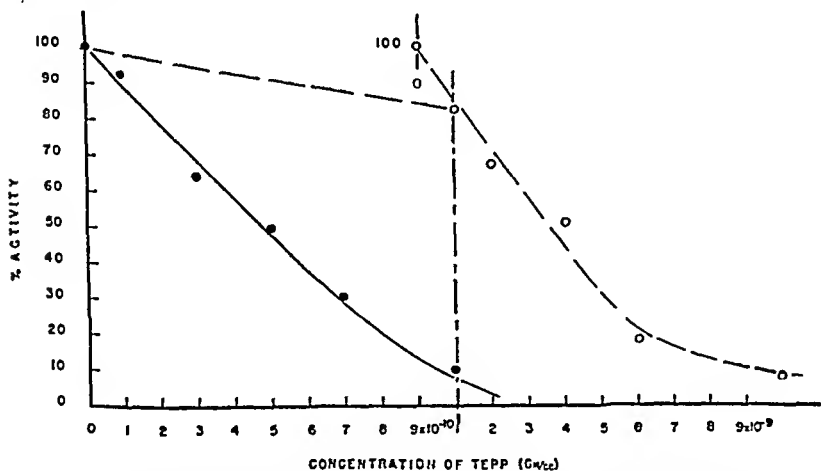


FIGURE 1. The relation between the concentration of tetraethyl pyrophosphate (TEPP) and the degree of inhibition of the cholinesterase activity of human plasma esterase (Fraction IV-6) ●, and of human erythrocyte stomata ○

resulting solution permitted to stand in the ice box for periods several times as long as required for the hydrolysis of 99% of the TEPP (9), little or no recovery of esterase activity could be demonstrated (Table 2). 2. The other test consisted in the dialysis of enzyme solution containing inhibitor against a larger volume

of inhibitor-free enzyme. This procedure obviates difficulties due to possibly very large distribution coefficients of TEPP between solutions which do and solutions which do not contain esterase. A typical result is presented in Table 3. No redistribution of the inhibitor, and no reactivation of the esterase could be detected.

The presence of TEPP can be detected by adding solutions suspected of containing the inhibitor to a solution of Fraction IV-6, and determining the residual esterase activity. All esterase activity in the test solutions must be destroyed

TABLE 2

Effect of storage on cholinesterase activity of plasma esterase treated with tetraethyl pyrophosphate (TEPP)

	CHOLINESTERASE ACTIVITY			
	After 1 hour		After 10 days in ice box	
	mM/L/hour	%	mM/L/hour	%
0.1% Fraction IV-6	1010	100	945	100
Same in 2×10^{-3} TEPP.	700	69	650	68
Same in 4×10^{-3} TEPP	380	37	375	39

TABLE 3

Effect of dialysis upon the cholinesterase activity of plasma esterase treated with hexaethyl tetrapolyphosphate (HETP) or with tetraethyl pyrophosphate (TEPP)

	% ACTIVITY		% ACTIVITY
Enzyme—HETP.	31	Enzyme—TEPP	48
Same after 24 hours dialysis against twice its amount of enzyme solution...	34	Same after 24 hours dialysis against twice its amount of enzyme solution	48
Same after 8 days.	34	Same after 5 days	45
		Same after 8 days	43
Calculated activity for uni- form distribution of HETP..	75	Calculated activity for uni- form distribution of TEPP.	83

by heating to 96°C. for 7 minutes before adding them to the indicator enzyme. This method was applied to the study of the interaction of TEPP and esterase.

Native or denatured Fraction IV-6 in 1% solution was mixed with enough TEPP to make a concentration of 10^{-8} . After this inhibitor solution had stood at 20°C. for 30 minutes, it was heated to 96°C. at pH 7.0 to 7.4 for 10 minutes to destroy all enzyme activity. This preparation was cooled and then diluted with 9 volumes of 0.01% Fraction IV-6 as indicator enzyme. The final concentration of TEPP, if it were not destroyed, would be 10^{-9} . The cholinesterase activity of this mixture was determined. From data illustrated in Figure 1 it is apparent that this method would detect about 10^{-10} of TEPP.

Denaturation of the esterase of Fraction IV-6 was accomplished by one of three methods: 1) 50% ethyl alcohol at 20°C.; 2) pH 1.0 for 15 minutes at 20°C.,

then neutralization to pH 7.4; and 3) 96°C. for 8 minutes. The results are presented in Table 4.

The activity of 0.01% Fraction IV-6 is taken as 100% (column 1). Since the indicator enzyme is added to one tenth of its volume of inactivated enzyme, 90% of the activity of Fraction IV-6 would be expected if no inhibitor activity remains in the inhibitor solutions. It appears that, whereas interaction of native esterase with TEPP resulted in the inactivation of the inhibitor (column 3), no such inactivation occurred when the esterase was denatured before coming in contact with TEPP (column 4). It was immaterial for this purpose whether the esterase was inactivated by heat, alcohol, or low pH.

In order to test whether simple molecular groupings could be formed, that were capable of interacting with TEPP, one per cent solutions of a number of

TABLE 4

Inactivation of tetraethyl pyrophosphate (TEPP) by native and denatured plasma esterase

METHOD OF DENATURATION	% CHOLINESTERASE ACTIVITY			
	Enzyme alone	Enzyme + TEPP	Enzyme + (TEPP + native 1% IV-6)	Enzyme + (TEPP + denatured 1% IV-6)
50% Ethyl Alcohol, 20°C.	100	2.5	88	3.6
pH 1.0, 20°C. neutralized to pH 7.4	100	7.0	86	7.5
96°C., 8 minutes..	100	0.9	95	1.8

The inhibitor solutions in all three series of experiments were subjected to the same treatment (96°C. at pH 7.0-7.4 for 10 minutes) before being cooled and diluted with 9 volumes of 0.01% Fraction IV-6 as the indicator enzyme. The final dilution of TEPP is $1:10^{-2}$ on the basis of the initial amount added. In all but the first column, 90% represents full activity of the esterase. In the presence of TEPP this value indicates concentrations of the latter less than 1×10^{-10} .

amino acids as well as other substances were brought in contact with TEPP under conditions of pH and ionic strength comparable to those employed in the interaction between the esterase and TEPP. In none of these experiments, however, was any significant decrease in the anti-esterase activity of the TEPP observed after periods ranging from one-half to one hour (Table 5, A). Inhibitor potency in these cases was compared with that of a solution of TEPP in pure buffer which had been allowed to stand for the same length of time as the experimental solutions.

The question was raised whether proteins other than those of Fraction IV-6 might be capable of inactivating TEPP. Table 5, B, shows the results of a study on various fractions resulting from the plasma fractionation process of Cohn, et al. (4)³. This indicates that no significant esterase inhibitor activity is lost on contact of TEPP with Fractions I and V (crystallized) whereas Fractions II + III, IV-1, IV-7, and IV-8 show a certain amount of activity, though less than Fraction IV-6.

Several mechanisms may be invoked to account for the inhibition of esterase

activity by neutral phosphate esters. The P moiety of the inhibitor might, for example, be bound to the enzyme. In order to test this possibility HETP containing P^{32} was prepared by reacting one mole of $P_2^{32}O_5$ with two moles of $OP(OC_2H_5)_3$ at $55^\circ C$. for 120 minutes, then at $100^\circ C$. for another 60 minutes (10). The compound was dissolved to give 50 cc. 0.1% solution in absolute ethanol. Its I_{50} was 2×10^{-9} M. One per cent solutions of Fraction IV-6-3 (purified about 10 times more than IV-6) were partially inactivated with this material, then dialyzed for 24 hours against running 0.9% sodium chloride solution. The solutions were deproteinized with ethanol, the precipitate separated in the cen-

TABLE 5
Inactivation of tetraethyl pyrophosphate (TEPP) by various substances

TEST SOLUTIONS	% CHOLINESTERASE ACTIVITY (0.01% FRACTION IV 6 = 100%) ^a
A. 10^{-8} TEPP in 0.9% NaCl solution	9
Same + 1% ethanol	7
" + 1% phenol	8
" + 1% n-propylamine	8
" + 1% guanidine	7
" + 1% tyrosine..	8
" + 1% cysteine	6
B. 10^{-8} TEPP in 0.9% NaCl solution	9
Same + 1% Fraction I	7
" + 1% " II + III	20
" + 1% " IV-1	38
" + 1% " IV-6	89
" + 1% " IV-7	38
" + 1% " IV-8	30
" + 1% " V (crystalline)	7

^a Nine volumes of 0.01% Fraction IV-6 were added to one volume of the test solution so that the final solution of TEPP (if none were destroyed) would be 10^{-9} . All solutions in group A were added to the enzyme without further treatment. Solutions in group B were treated as described under Table 4 before addition to the 0.01% Fraction IV-6.

trifuge, and the supernatant evaporated to dryness on the steam table after digestion at pH 10. In trial runs without the addition of enzyme, 80% or more of the added radioactivity could be recovered from the solution by this procedure. Four experiments were performed with enzyme in this fashion. Sufficient radioactive material was added to the enzyme to give 300 to 600 counts per minute with the counter geometry employed. In no case was any significant amount of radioactivity (0 to 0.7% of the total radioactivity added) recovered from either the deproteinized supernatant or the protein precipitate obtained from the dialyzed preparation. Table 6 shows the protocol of a typical experiment in which an excess of enzyme was added. Observation 2 shows that HETP at a concentration of 10^{-6} gm. per cc. inhibited 35% of the cholinesterase activity of the enzyme. Observations 2a and 2b show that when the material of observation 2

was heated and tested for residual HETP by means of indicator enzyme (as described under Table 4), no inhibition could be detected; hence the concentration of active HETP in the original preparation 2 is less than 10^{-8} . Observation 3 confirms the irreversibility of the inhibition of plasma esterase under the conditions of these experiments (cf. Table 3), and demonstrates the absence of P^{32} from either the protein precipitate or the supernatant. Observations 4 and 5 show that no binding of P^{32} occurs when heat denatured Fraction IV-6-3 was used in place of the native enzyme. Observations 4a and 4b indicate that in-

TABLE 6

The reaction between hexaethyl tetrapolyphosphate (HETP) containing p^{32} and plasma esterase

OBSERVATION	CHOLINESTERASE ACTIVITY		COUNTS/MINUTE
	mM/L/hr	%	
(1) 1% solution of Fraction IV-6-3 in 0.9% NaCl solution.	80,000	100	0
(2) (1) in 10^{-6} Gm./cc. HETP*..	52,000	65	520
(3) (2) after 24 hours dialysis against running saline (2 cc./min.) at 20°C.	50,000	63	
Protein precipitate			0
Supernatant.			0
(4) (1) heated to 96°C. for 5 min., cooled, + 10^{-6} Gm./cc. HETP†	0	0	520
(5) (4) after 24 hours dialysis against running saline (2 cc./min.) at 20°C.	0	0	
Protein precipitate			2
Supernatant.			2
* (2a) (2) heated to 96°C. for 5 min., diluted with 9 volumes of 0.01% Fraction IV-6.	67		
(2b) 0.009% Fraction IV-6.	70		
† (4a) (4) heated to 96°C. for 5 min., diluted with 9 volumes of 0.01% Fraction IV-6.	4.6		
(4b) Same as (2b).	70		

hibitor remained in the material of observation 4 in a concentration equivalent to more than 10^{-7} gm./cc. of HETP.

Experiments were also performed which were identical with the above except for the omission of the dialysis. Again no radioactive material could be detected in the protein precipitate washed twice with 3 cc. of 50% ethanol.

DISCUSSION: The reaction between plasma esterase and several neutral phosphate esters can be described in some detail on the basis of the experiments reported above.

a. *Structural Requirements.* Examination of the data of Table 1 reveals that phosphorus compounds of many different structures are active esterase inhibitors. The only feature common to all of the active compounds is the presence of the grouping $\equiv P-O-R$ where R may be an alkyl or an aryl radical. On the other hand, the presence of this grouping alone is not a sufficient condition

for anti-esterase activity. This can be illustrated by the following pairs of compounds: 1) triethyl phosphate—tetraethyl pyrophosphate; 2) trimethyl phosphate—dimethyl fluorophosphate (1); 3) tri p-cresyl phosphate—tri o-cresyl phosphate (2). In each case the first member of the pair is inactive, while the second is active as an esterase inhibitor. It may be worth noting that in each pair only the active compound contains an arrangement which would be expected to have a high free-energy content. In pair 1 this feature is the pyrophosphate linkage, in pair 2 it is the analogous anhydride of hydrofluoric and dimethyl phosphonic acids, and in pair 3 it is the steric strain which probably exists in the tri o-cresyl phosphate.

b. *Reversibility.* Figure 1 shows that the relation between the concentration of TEPP in solutions of Fraction IV-6 and the degree of inhibition produced is linear over a wide range. Such a relation can be explained most readily in terms of stoichiometric relation between the number of active enzyme centers destroyed and the number of inhibitor molecules present, and by the further assumption that inhibition, once it is produced, is virtually irreversible under the conditions of these experiments. This hypothesis was tested by dialysis experiments as well as by experiments in which the enzyme solutions containing inhibitor were stored under such conditions that virtually all of the inhibitor would be hydrolyzed. The results in both cases support the assumption of an irreversibility of the esterase inhibition under the conditions of this study. This does not mean that it may not be possible, under certain other circumstances, to regenerate the activity of plasma esterase inactivated by HETP or by TEPP. Indeed, certain preliminary observations indicate such a possibility *in vivo*. Thus, canine plasma esterase behaves *in vitro* like human plasma esterase with respect to its sensitivity to HETP as well as with regard to the failure of the inhibited enzyme to recover on storage or on dialysis. Yet, following HETP administration *in vivo* the plasma esterase activity of dogs recovers several times more rapidly than after comparable doses of di-isopropyl fluorophosphate; this difference cannot be accounted for in terms of different effects of the two agents upon the hepatic plasma esterase store.

c. *Reaction of HETP with Human Plasma Cholinesterase.* It was shown that on interaction with esterase the inhibitor is inactivated as well as the enzyme. This is true for HETP as well as for intact enzyme. Denaturation of the enzyme, whether by ethanol, by heat, or by low pH, prevents the destruction of added inhibitor. Several other pure proteins behave like denatured esterase in this respect, while fractions containing high globulin concentrations show some ability to inactivate TEPP, although less than the main esterase bearing fractions. A detailed study of the distribution of phosphoric acid esterases in the plasma protein fractions used in this study has not yet been carried out. However, the possibility was considered that the inactivation could represent an enzymatic destruction of HETP similar to that observed by Mazur (11) for DFP. Such an interpretation was excluded on the following grounds: (a) Fraction IV-6 (0.1%) does not noticeably accelerate the hydrolysis of 0.01–0.2 M solutions of HETP (determined manometrically); (b) 0.01 M Hg^{++} which completely

inactivates Mazur's Enzyme, does not prevent the interaction between HETP (or TEPP) and Fraction IV-6; (c) there appears to be a stoichiometric relation between the amount of inhibitor inactivated and the concentration of purified plasma esterase. It is possible therefore that the ability of a protein to inactivate TEPP or HETP is, in a measure, tied to the presence of groupings and of the configuration which impart cholinesterase activity to that protein.

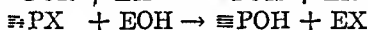
An attempt was made to shed further light upon this reaction by the use of HETP containing P^{32} . The method of preparation chosen was intended to yield a product with a statistically uniform distribution of P^{32} among all the phosphorus of the molecule. The tests described above show that a stable compound between the enzyme and a phosphorus-containing moiety of the inhibitor is not formed. Any compound of this type that may be formed as an intermediate cannot survive ethanol precipitation of the protein at room temperature, nor can it survive 24 hours of dialysis of the enzyme against 0.9% sodium chloride solution at room temperature.

The biological differences in the behavior of plasma esterase inhibited by DFP on the one hand, or by HETP or TEPP on the other hand, may be recalled in this connection. These observations may be interpreted as indicating the formation of different end products as a result of the interaction of plasma esterase with the two types of inhibitors.

d. *Mechanism of Inhibition.* A working hypothesis which is not in contradiction to any of the facts uncovered thus far can be formulated as follows:



or



where $\equiv\text{POR}$ or $\equiv\text{PX}$ is the inhibitor, EH or EOH the active enzyme, ER or EX the inactivated enzyme, R an alkyl or an aryl group, and X a halide.

This hypothesis is now being tested both with regard to the proposed scheme of reaction, and with regard to the groups of the enzyme which participate in the reaction.

SUMMARY

1. A series of alkylated phosphorus compounds has been tested in vitro for their esterase inhibiting effects. The grouping $\text{P}-\text{O}-\text{R}$ (R alkyl or aryl) is common to all active compounds. Other structural requirements are pointed out.

2. Plasma esterase inactivated by hexaethyl tetrapolyphosphate (HETP) or by tetracthyl pyrophosphate (TEPP) cannot be reactivated by the removal of the inhibitor in vitro, either by dialysis against active esterase for 24 hours, or by storage under conditions resulting in the destruction by hydrolysis of the inhibitor.

3. Interaction of HETP or TEPP with plasma esterase leads to the inactivation not only of the enzyme, but also of the inhibitor. Denatured esterase does not inactivate the compounds. No simple molecular groupings were found capable of interacting with TEPP at a rate approaching that involved in its reac-

tion with enzyme. Fibrinogen and crystalline albumin obtained from human blood plasma did not show any significant interaction with TEPP.

4. HETP containing P^{32} was prepared. It could be shown that upon interaction of this compound with plasma esterase no P^{32} could be found in the protein precipitated by ethanol, nor in the precipitate or in the supernatant of such preparations after dialysis. It was concluded that a stable combination between the esterase and a phosphorus containing moiety of HETP does not take place to a detectable degree.

5. On the basis of these data a working hypothesis has been proposed to describe the interaction of plasma esterase with inhibitors of the type here considered.

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ON THE PHARMACOLOGY OF HEXAETHYL TETRAPHOSPHATE¹

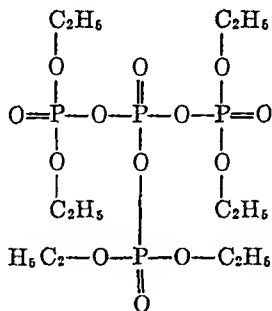
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The close of World War II saw a great number of chemical compounds brought over from Germany into this country by the Technical Intelligence Committee. One of these, hexaethyl tetraphosphate (HETP), was used by the Germans as an insecticide having, according to its original users, a nicotine-like action. It was for this purpose that HETP was first introduced into this country. We became interested in this compound following a demonstration of its muscarinic actions by Drs. V. K. Rowe and D. D. Irish of the Dow Chemical Co. in their laboratories in December, 1946.

HETP ($C_{12}H_{30}P_4O_{13}$) is a viscid liquid with a specific gravity of 1.29, a light brownish tint, and a characteristic aromatic odor. It is freely miscible with water, but its aqueous solutions deteriorate very rapidly, hardly any of their potency remaining after 24 hours. The following structural formula has been assigned to it:



The first paper on HETP to appear in this country was that of DuBois and Mangun (1) which reported that animals injected with this drug exhibited symptoms similar to those produced by Di-isopropyl Fluorophosphate (DFP), namely, muscular twitchings, tonic and tonic-clonic convulsions, involuntary defecation, micturition and salivation, and miosis upon local instillation of the drug into rabbit's eyes. Subsequent experiments *in vitro* and *in vivo* showed that these symptoms could, to a great extent, be explained by a cholinesterase inhibiting action of HETP. In the present work, an attempt was made to investigate

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these actions further and to determine how closely the effects and manner of action of HETP paralleled those of DFP, with the ultimate end in view of assessing the possible value of the drug in the treatment of various clinical conditions requiring an anticholinesterase compound.

EFFECTS ON INTACT UNANESTHETIZED DOGS: Twenty-two intact unanesthetized dogs were given various doses of HETP by the intravenous and intramuscular routes, and their symptoms observed. The HETP was prepared as a 1 per cent solution in distilled water, fresh solutions being made up just before use to avoid deterioration. Fifteen dogs received single injections of the drug, the other seven, repeated doses.

The general effects of HETP are a combination of muscarinic and nicotinic signs, consisting of diarrhea, tenesmus, borborygmus, vomiting, retching, salivation, micturition, dyspnea, muscular fasciculations, tremors, weakness, ataxia, and terminal convulsions of the clonic or tonic-clonic type. The muscarinic effects, particularly those of the gastrointestinal tract and bladder, dominated the picture in the lower dose ranges (0.1-0.4 mgm. per kgm. i.v.), very little nicotinic effects being observed with these doses. With larger doses (0.8 mgm. per kgm. or more, i.v.), muscle fasciculations, tremors, weakness and ataxia were more prominent and increased in severity with increasing dosage, convulsions usually ushering the death of the animal. No definite sequence in the onset of these various signs was observed, although with the larger doses, it was more common to see fasciculations of the muscles of the neck, back and thigh appearing before the other manifestations.

A few dogs showed evidence of pilomotor stimulation. The effect on the pupil was not prominent, some degree of miosis being noted with large doses (more than 1 mgm. per kgm.). The heart rate changes from the smaller doses of HETP were not constant: a moderate decrease, increase, or no change at all being observed with different animals. However, doses large enough to cause marked nicotinic effects produced a marked fall in the ventricular rate to 60 per minute or thereabouts; and lethal doses of the drug (about 1.3 mgm. per kgm. intravenously or 1.5 mgm. per kgm. intramuscularly) uniformly produced slowing to 40 or less per minute.

As on the pulse rate, HETP apparently did not have definite effects on the respiration in the lower dose ranges; but with the higher dosages, the animals exhibited marked dyspnea and tachypnea (shown later to be due mostly to bronchoconstriction). In the animals that succumbed, death was due to respiratory failure, the heart continuing to beat for some time after cessation of respiration.

When given intravenously, HETP produced its effects within 1 to 2 minutes. The signs rapidly increased in severity becoming maximal within 5 to 10 minutes depending on the size of the dose. These effects were fairly brief, starting to subside in 1-2 hours, and 2-4 hours after the injection of the drug, the animal appeared essentially normal. When lethal doses of HETP were administered, the onset of the effects was much more abrupt, the animal rapidly becoming too weak to raise its head. Generalized tremors merged with tonic-clonic con-

vulsions and death ensued within 10 to 15 minutes. The latent period and duration of action were somewhat longer when the drug was injected intramuscularly, and slightly larger doses were required to produce effects comparable to the intravenous dose, but in hardly any instance did the effects persist longer than 5-6 hours, nor was death, from toxic doses, delayed more than 1-2 hours. Animals remaining alive after this time eventually recovered. This uniformly brief observable period of action of HETP as well as its rapidly lethal effect are characteristic of this drug, and differ in this respect from DFP, whose effects last from 6 hours to 17 days, and from which death can occur over almost the same range of time (4 minutes to 16 days) (2).

In 7 dogs where a second injection of HETP was given 24 hours after the first dose, additive effects were produced. The animals had all completely recovered from the visible effects of the priming dose when the second injection was administered.

EFFECTS ON ANESTHETIZED DOGS. *Experimental.* Eighteen dogs, weighing 7-18 kgm., were anesthetized with pentothal sodium-barbital sodium intravenous sequence anesthesia. The trachea was cannulated in all animals, and artificial respiration by means of a respiratory pump was given to 5 animals. The vagi were isolated for sectioning. The carotid artery blood pressure was recorded by means of a mercury manometer; the respiration, by means of a chest pneumograph. The degree of broncho-constriction was measured by means of a Rossler apparatus. Femoral flow studies by means of a differential pressure cannula and capsula were done on 2 dogs. Lead II was used for electrocardiographic studies. Newly prepared solutions of HETP were used in all instances, and the injections were administered into the exposed femoral veins.

Effects on the circulation. The anesthetized dog was found to be more sensitive to HETP than the intact unanesthetized animal. Doses of HETP sufficient to cause bradycardia and changes in the blood pressure in the anesthetized animal were 15-30 per cent less than those producing similar effects in the unanesthetized dog.

The blood pressure was not significantly altered by small doses of HETP. Such doses (0.5 mgm. per kgm. or less) produced no change in the blood pressure in some animals, a slight transient fall in others, and more usually (in about 50 per cent) a transient rise of 5-10 mm. mercury. The fall in the blood pressure was most probably due to muscarinic action of the drug on the blood vessels. The blood pressure rise was a little more difficult to explain. Accordingly, femoral flow studies were done and it was found that with the rise in the arterial pressure, there was a concomitant increase in the blood flow through the leg. It is well known that epinephrine injected intravenously causes a similar effect of blood pressure and increased femoral flow. Since HETP, as shown by DuBois and Mangun and from data presented here, has an anticholinesterase action, a stimulation of the adrenals with consequent release of epinephrine into the blood stream would seem to be the most logical explanation for this phenomenon.

These small doses had inconstant and variable effects on the heart rate. In some animals the rate was unchanged, in others there was a slight acceleration or deceleration of about 5-10 beats per minute.

After larger doses of HETP (0.5-0.8 mgm. per kgm.), a slowing of the heart rate became more manifest; initial rates of 150 or 200 dropped down to about 90 or 100 per minute. But even with these doses, the effect on the blood pressure was unpredictable. A few animals showed no blood pressure changes at all; others responded with the same fleeting elevations or depressions observed with the small doses; while still others had a gradual fall in blood pressure amounting to 20 mm. mercury or more over a period of 30-60 minutes.

With still larger doses (0.8-1.0 mgm. per kgm.) a point was suddenly reached at which the animal started to exhibit deep and labored breathing and marked bradycardia of 30-60 per minute. In 3 animals, this point was reached after only 0.6 mgm. per kgm. With the development of these profound effects of the drug, marked blood pressure changes occurred. There was usually an initial blood pressure rise, marked in some dogs, slight in others, which is probably asphyxial since it occurred with the development of marked bronchoconstriction (see below). This was promptly followed by a rapid fall to shock levels. The integrity of the vagus nerves had no effect on the development of these manifestations, but atropine, as little as 0.1 mgm. per kgm. promptly caused acceleration of the heart rate, disappearance of the dyspnea and a rise in the blood pressure back to practically its original level.

Electrocardiographically, the marked slowing of the heart rate was seen to be due either to a marked sinus bradycardia or more usually to A-V block, varying in grades from partial to complete.

In the animals which were able to maintain a sufficient respiratory exchange, or in those to whom artificial respiration was administered, death from these toxic doses could be prevented for as long as 1-2 hours, without resorting to atropinization, although the blood pressure was at near-shock levels (30-50 mm. mercury) and the state of A-V block continued. Occasionally, dogs with low grade heart blocks were able to recover and to resume spontaneously their normal sinus rhythm. When however, after these large doses, the respirations ceased, as they often did at this stage of poisoning, the heart went into ventricular fibrillation and death quickly followed.

Electrocardiographic findings. The electrocardiographic changes due to HETP, as observed in the anesthetized unprotected animals, have been mentioned in part. The P-R interval was not prolonged by doses of HETP which did not produce the profound A-V conduction defects just described. In other words, the effect of HETP on the A-V conducting system seemed to follow an all-or-none principle; or more accurately, this probably means that the difference between the doses producing simple conduction delay and partial or complete heart blocks is so small that we were unable to hit upon the critical dose at which A-V conducting system just started to show some depression.

Toxic doses of HETP produced complete auriculo-ventricular dissociation, sino-auricular node block with disappearance of the P waves, and idioventricular rhythm. All these changes were promptly reversed by atropine. No other constant characteristic effects were observed in the electrocardiographic pattern even after the injection of several lethal doses of HETP into the atropinized dog.

Effects on the respiration and bronchi. In unprotected dogs, HETP in large doses caused death by respiratory failure. This was preceded by a period of labored respiratory effort and increased rate. Smaller doses produced only this latter phase of respiratory stimulation, while doses sufficient to cause strong vagal stimulation resulted in eventual cessation of breathing prior to cardiac arrest. As little as 0.6 mgm. per kgm. of HETP was effective in bringing about definite bronchoconstriction, and doses of 1–1.3 mgm. per kgm. constricted the bronchi to the point of extreme resistance to passage of air. Aside from the bronchoconstriction, increase in the mucous secretion probably plays a not unimportant rôle in interfering with effective respiration. Partial autopsy on 2 dogs which died with severe respiratory embarrassment revealed marked mucoid

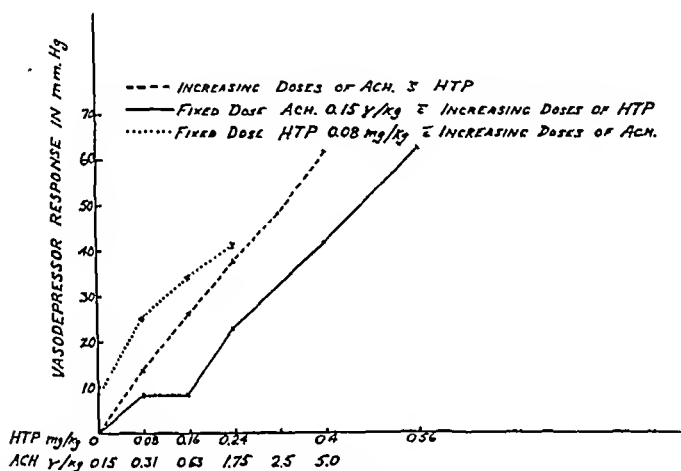


FIG. 1. POTENTIATION BY HETP OF THE VASODEPRESSOR ACTION OF ACETYLCHOLINE

secretion throughout the bronchial tree. Atropine, 0.1 mgm. per kgm. was an effective antidote for the bronchoconstriction and dyspnea. Animals so protected were able to tolerate several lethal doses of HETP.

Acetylcholine potentiation. Both the vasodepressor and, after atropinization, the vasopressor actions of acetylcholine were potentiated by HETP. Fig. 1 shows a striking parallelism between the vasodepressor effects of graded doses of acetylcholine alone and those of a small fixed dose of acetylcholine potentiated by graded ascending doses of HETP. A small initial dose of HETP intensified the effects of all subsequent doses of acetylcholine, although the larger doses were not potentiated as much as the smaller ones.

Atropinization produced a reversal in the acetylcholine response. These pressor responses to acetylcholine were also markedly potentiated by HETP (fig. 2).

The duration of this potentiation was prolonged, lasting at least 7–8 hours.

PROTECTION AGAINST HETP TOXICITY. The effectiveness of atropine as an antidote against the toxic and lethal actions of HETP was studied in 18 intact unanesthetized dogs (table 1).

Atropine protective action was exerted principally against the muscarinic action of HETP. The dose of atropine necessary to afford complete antagonism of these effects varied with the HETP dose. With one or two lethal doses of HETP (1.3–3.0 mgm. per kgm.), 0.1 mgm. per kgm. of atropine was adequate in reversing most of the parasympathetic signs; larger doses of HETP required correspondingly larger doses of atropine. The varying degrees of effectiveness with which atropine blocks the parasympathetic effects on various organs also

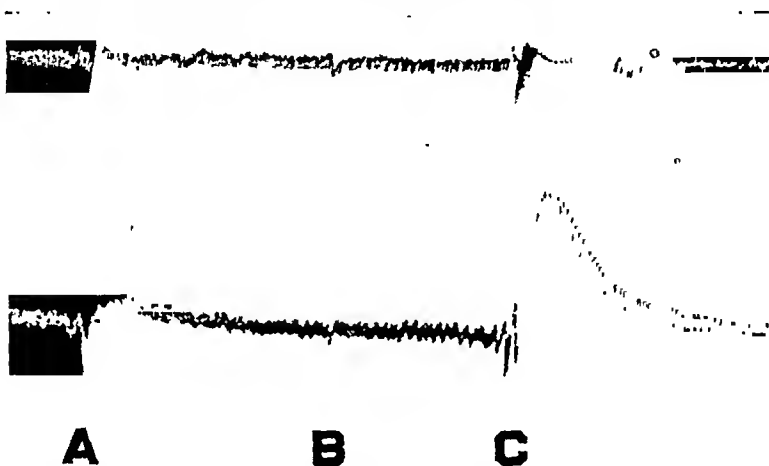


FIG. 2. POTENTIATION BY HETP OF THE VASOPRESSOR ACTION OF ACETYLCHOLINE
A. Acetylcholine 0.25 mgm. per kgm. i.v. B. HETP, 0.1 mgm. per kgm. i.v. C. Acetylcholine, 0.25 mgm. per kgm. i.v. Upper tracing represents respiration.

held true with this drug. The vagal actions on the heart and the bronchial muscles, for instance, responded promptly and completely to atropinization, while the stimulant effects on the gastrointestinal tract and the bronchial glands required much larger doses of atropine. The lethal action of HETP in dogs A-12, A-14, and A-20 could probably be ascribed to the inadequate protective dose of atropine given, and the consequent plugging of the bronchial passages with mucous. These animals exhibited marked dyspnea and audible mucous rales. Increasing the dose of atropine to 0.5 mgm. per kgm. enabled dogs A-15 and A-21 to tolerate 5.0 mgm. per kgm. of HETP.

Atropine was able to protect dogs from approximately three lethal doses of HETP, (5.0 mgm. per kgm.). Ten mgm. per kgm. doses of HETP were rapidly fatal even when given with doses of atropine large enough to eliminate all muscarinic effects.

The promptness of recovery after atropinization depended upon the size of the HETP dose. After one lethal dose, atropine enabled the animal to recover very promptly, with only a few residual nicotinic signs. A prostrated dog with

TABLE I
Protective action of atropine against HETP toxicity

DOG NO.	HETP		ATROPINE			REMARKS
	Dose	Route	Dose	Route	Time of injection	
	mgm / kgm.		mgm / kgm.			
1	1.3	i.v.	1.0	i.v.	During the height of the effects	Immediate recovery
2	1.3	i.v.	1.0	i.v.	During the height of the effects	Immediate recovery
3	1.3	i.v.	0.1	i.v.	During the height of the effects	Immediate recovery
4	1.3	i.v.	1.0	i.v.	After respiratory arrest	Died
5	1.5	i.v.	1.0	i.v.	After respiratory arrest	Died
6	2.0	i.v.	2.0	i.v.	Before HETP injection	Developed only a few nicotinic signs
A-7	2.0	i.m.	0.1	i.m.	During the height of the effects	Recovered after about 2 hours
A-11	3.0	i.m.	0.1	i.v.	During the height of the effects	Recovered after more than 2 hours
A-12	5.0	i.m.	0.1	i.m.	During the height of the effects	Died
A-14	5.0	i.m.	0.1	i.v.	During the height of the effects	Died
A-20	5.0	i.m.	0.3	i.m.	Before HETP injection	Died
A-15	5.0	i.m.	0.5	i.v.	During the height of the effects	Recovered; severe effects started to subside in 2½ hours.
A-18	5.0	i.m.	0.5	i.m.	Same time as the HETP injection	Recovered; severe effects started to subside in 3½ hours.
A-21	5.0	i.m.	0.5	i.m.	Before HETP injection	Recovered; nicotinic signs not very severe
A-16	10.0	i.m.	0.2	i.m.	Before HETP injection	Died
A-17	10.0	i.m.	1.0	i.m.	Before HETP injection	Died
A-19	10.0	i.m.	2.0	i.v.	Just after HETP	Died
A-22	10.0	i.m.	5.0	i.m.	Before HETP injection	Died

bradycardia, dyspnea, tremors and convulsions, can thus within a few minutes be able to stand up and walk about practically symptom-free. Larger doses of HETP, on the other hand, caused such severe nicotinic effects that, even after the heart rate had been accelerated to a tachycardia and the labored breathing

had been eased by atropine, the marked weakness, generalized tremor and convulsions persisted for 2 hours or longer, and the dog did not recover completely for several hours (5-6 hours). Death in these cases invariably occurred during the first one or two hours following the HETP injection.

This experiment makes possible the evaluation of the relative importance as a cause of death of the two types of actions of HETP. In the smaller doses of the drug, the muscarinic actions predominated and death from this mechanism occurred after one or two, or probably even three lethal doses. Death came as a result of respiratory and cardiac failure, with the former playing the more important rôle. Cessation of respiration always preceded cardiac arrest. Atropine, as has been shown above, efficiently protected against this type of death. It was only in the very last stages of poisoning, after the respirations had ceased, that atropine even with artificial respiration could not revive the animal (Dogs 4 and 5).

The nicotinic action, which came to the fore after the muscarinic effects had been blocked, likewise resulted in respiratory failure presumably from direct central depression by the accumulated acetylcholine, or the curariform effect of acetylcholine on the skeletal muscles, or both. Death from this mechanism occurred only with very large doses of the drug (10.0 mgm. per kgm.). Artificial respiration appears to provide added protection against this type of death. In acute experiments, atropinized and artificially respired dogs were able to tolerate more than 32 mgm. per kgm. of HETP.

Koster (3) has reported the interesting finding of protection from DFP toxicity in cats by the previous injection of small doses of physostigmine. Brauer and his co-workers (4) reported such protection against HETP toxicity in rats. We failed to observe a similar protection by physostigmine in mice.

Acute toxicity. The LD_{50} of HETP in albino mice by intraperitoneal administration as calculated by Behren's method is 6.1 mgm. per kgm. This value is obviously very much larger than the lethal dose in the dog.

MIOSIS IN RABBITS. Miosis was not a prominent sign in dogs given parenteral injections of HETP. However, local instillation of dilute solutions into the conjunctival sac, as DuBois and Mangun have shown (1), caused early but brief miosis. In this work, 2 drops of a 0.25 per cent solution of HETP in physiologic saline were instilled into the right conjunctival sac of several rabbits, the other eye being used as control. In each instance, miosis was observed to appear within 3 minutes, was maximal in 5 minutes, started to diminish in about 3 hours and passed off completely in 12-24 hours. No signs of irritation were observed. Control instillations of physostigmine salicylate, 0.25 per cent solution, were found to have similar effect, the only difference being that the duration of the maximal action was only 1 hour.

One per cent solution of atropine sulfate, instilled locally, is an effective antagonist against HETP miosis, although not as promptly effective as against physostigmine. Atropine could completely counteract physostigmine miosis within 15 minutes; against HETP, 2-3 hours were required before complete recovery.

CHOLINESTERASE INHIBITION. The work of DuBois and Mangun on rats (1) has established the cholinesterase inhibitory action of HETP *in vitro* and *in vivo*. In the following experiments, we extended the study to *in vivo* work in dogs.

Methods. Seven dogs were injected intravenously and 2 dogs intramuscularly with varying doses of HETP. Blood samples were taken from each animal before the injection of the drug, 10–15 minutes after the injection during the height of the effects, and then periodically thereafter for several weeks. Immediately after extraction, the blood was defibrinated by stirring, centrifuged, and the serum and red blood cell cholinesterase (ChE) activities determined by the method of Ammon (5) as modified by Mazur and Bodansky (6). The serum was diluted 1:5 with 0.025 M NaHCO₃. The red cells were washed twice with 5 times their volume of physiologic salt solution, and then hemolyzed with 3 times their volume of 0.025 M NaHCO₃. One-half cc. of each sample of diluted serum and hemolyzed red cells was determined for its enzyme activity. Corrections were made for the non-enzymatic hydrolysis of acetylcholine. The straight line part of the reaction curve was taken

TABLE II
Effect of HETP on blood ChE activity

DOG NO.	HETP	ROUTE	CHOLINESTERASE ACTIVITY	
			Serum	Erythrocytes
	<i>mgm./kgm.</i>		%	%
G	0.4	i.v.	11.7	38
B	0.8	i.v.	0.0	7
E	0.8	i.v.	4.0	8
F	1.0	i.v.	9.0*	4
H	1.0	i.m.	0.4	—
D	2.0	i.v.	0.0	0
I	2.0	i.m.	8.0	9

* Some hemolysis present in this sample.

In all instances, the volume of CO₂ in c.mm. produced in 30 minutes by 1 cc. of serum or 1 cc. of packed red blood cells was calculated, and the values expressed as per cent of the ChE activity of the control samples.

Effects on blood cholinesterase. The initial depression of the esterase activities of both the serum and red cells attest to the marked potency of HETP as a cholinesterase inhibitor. These samples were taken during the height of the effects, i.e. 10–15 minutes after injection of the drug. The results are shown in table 2.

In agreement with previous work on DFP (6, 7), the serum was found to be more sensitive to the enzyme inhibitor than the red cells. This is probably due to the fact that serum esterase is largely non-specific as Mendel and Rudney (8) have shown. This fact may also explain the closer relationship between the intensity of the manifestations and the red cell enzyme activity. Dog G, for instance, which was injected with 0.4 mgm. per kgm. of HETP, developed only mild muscarinic signs and minimal nicotinic effects. Its ChE activity at this time was depressed to 11 per cent of normal, a level almost as low as those pro-

duced by doses of HETP (0.8 mgm. per kgm. or more) which effected marked nicotinic signs. In the same animal, the erythrocyte ChE activity was depressed to only 38 per cent of normal, sufficiently high to be compatible with the minimal effects observed. The *in vitro* studies of Koelle and Gilman (7) which established the same order of susceptibility between erythrocyte, brain and muscle cholinesterases, also called attention to this relationship between the erythrocyte enzyme activity and the symptoms.

The recovery of the enzyme activities of both the erythrocytes and the serum was markedly prolonged (figs. 3 and 4). The serum activity, however, showed a more rapid return to normal. Dog D attained normal serum esterase activity within 9 days, and Dog B within 16 days, at which times, their erythrocyte levels were only 67 and 81 per cent respectively. On the other hand, Dog A had not

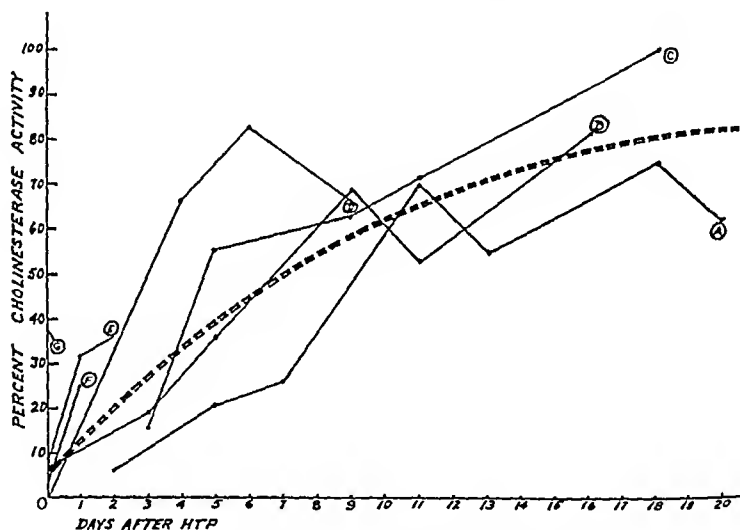


FIG. 3. DOG ERYTHROCYTE CHOLINESTERASE RECOVERY CURVE

recovered full enzymatic activity 41 days after the injection, its serum level being 90 per cent and its erythrocyte activity 70 per cent.

A study of the mean recovery curves of the serum and red cells brings out the following points: The red cell recovery curve, although flatter than that of the serum, follows the same parabolic form. The initial recovery, particularly of the serum, is rapid, 70 to 80 per cent levels being attained during the first week. The red cells do not recover as promptly, although during the first 24-48 hours, they also exhibit a marked return of activity—from 7 to 32 per cent in Dog E, and from 4 to 25 per cent in Dog F. Full restoration to normal activity takes several weeks.

Attempts to attribute the briefness of the HETP effects to a prompt initial recovery of the ChE activity during the first few minutes or hours following the injection gave negative results. Blood withdrawn during the height of

the drug action and after the effects have worn off (1-2 hours later) did not show any significant difference in the enzyme activity of either the red cells or the serum.

The combination between the cholinesterase and HETP is apparently largely irreversible. Dialysis against physiologic saline of dog serum treated with various concentrations of HETP (1×10^{-6} to 1×10^{-3}) failed to restore its activity even after 24 hours.

Effects on Brain Cholinesterase. Since the erythrocyte studies did not furnish any explanation for the shortness of the observed effects of the drug, we were prompted to make enzyme studies of the brain, which according to Mendel and Rudney (8) contained only "true cholinesterase."

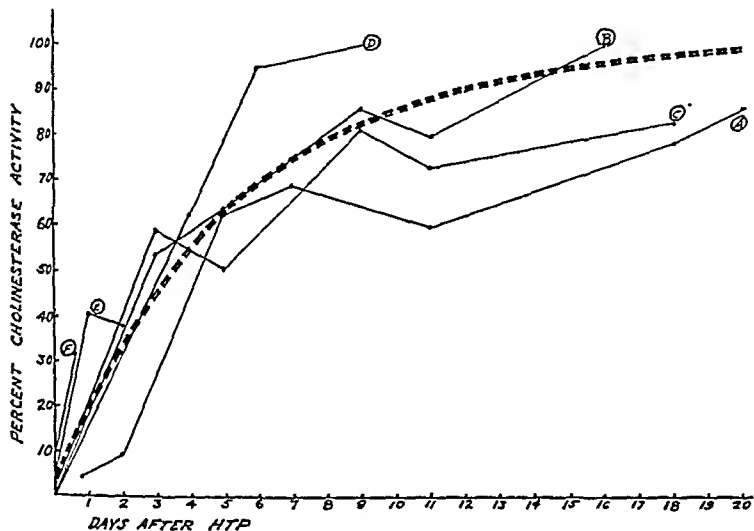


FIG. 4. DOG SERUM CHOLINESTERASE RECOVERY CURVE

Methods. Sixteen dogs were injected with different doses of HETP. Some of these animals were not atropinized and allowed to die; the others were protected with atropine and sacrificed at varying periods after the injection. The brain was rapidly removed after death, the pia mater and surface blood vessels stripped off as completely as possible, and the whole brain homogenized in a Waring blender with four times its volume of 0.025 M NaHCO_3 . One-half cc. of this homogenate was measured manometrically for its enzyme activity, following the procedure previously outlined. The volume of CO_2 in c.mm. produced by 1 gm. of brain in 30 minutes was calculated and expressed as per cent ChE activity of normal, the average of 5 normal brains.

Thirty CFW strain mice, weighing 18-22 gm., were injected intraperitoneally with 3 mgm. per kgm. ($\frac{1}{2}$ LD₅₀) of HETP. These were divided into three groups and sacrificed after 15, 60 and 120 minutes. Another group of 10 untreated mice served as control. The ten brains in each group were pooled, homogenized and their enzyme activity determined as described above.

The only significant brain ChE recoveries observed during the first 2-4 hours that might account for the briefness of the drug effects were in dogs given 1.5

mgm. per kgm. (table 3). In this group, the control animal (A-1), died 15 minutes following the injection, and at this time its brain ChE activity was only 18 per cent of normal. Dogs A-9 and A-8 which were sacrificed after they were well on their way to recovery (i.e. 3 and 4 hours after the HETP injection) showed brain activities of 45 and 33 per cent respectively. The animals injected with larger doses of HETP did not show any such prompt recuperation of their enzymic functions, although they had almost completely recovered before they were sacrificed (Dogs A-13, A-15 and A-18).

The preliminary work on mouse brain cholinesterase furnish additional suggestive evidence of an initial rapid regeneration. However, more data is needed

TABLE III
Dog brain ChE recovery after inhibition by HETP

DOG NO.	HETP	BRAIN ChE ACTIVITY	REMARKS
	mgm./kgm.	per cent	
A-5	1.0	49	Sacrificed $\frac{1}{2}$ hour after injection
A-2	1.0	42	Sacrificed $5\frac{1}{2}$ hours after injection
A-3	1.0	69	Sacrificed 3 days after injection
A-4	1.5	18	Died 15 minutes after injection
A-9	1.5	45	Sacrificed 3 hours after injection
A-8	1.5	33	Sacrificed 4 hours after injection
A-1	1.5	40	Sacrificed 24 hours after injection
A-10	2.0	11	Died 54 minutes after injection
A-13	2.0	12	Sacrificed $2\frac{1}{2}$ hours after injection*
A-7	2.0	53	Sacrificed 4 days after injection*
A-11	3.0	81	Sacrificed 18 days after injection*
A-12	5.0	4	Died 15 minutes after injection
A-14	5.0	7	Died 12 minutes after injection
A-15	5.0	7	Sacrificed $2\frac{1}{2}$ hours after injection*
A-18	5.0	4	Sacrificed $3\frac{1}{2}$ hours after injection*
A-21	5.0	35	Sacrificed 20 hours after injection*

* Adequately atropinized.

before it can be definitely stated that the differences now observed are statistically significant.

Discussion. HETP is a potent anticholinesterase drug whose effects can largely be explained by its action in inhibiting this enzyme. Judging from experiments *in vitro*, this action is apparently irreversible.

However, a careful study of the *in vivo* effects of HETP as compared with those of DFP shows some important differences between the two drugs: (1) The effects of HETP are relatively much briefer, lasting only a few hours at the most, while those of DFP last for several days and may even result in permanent injury. (2) The lethal action of HETP occurs within 1-2 hours, animals not dying within this period recovering eventually; from DFP, death may occur as long as 4 days after the injection. (3) HETP miosis is likewise relatively brief; DFP causes miosis lasting several days. (4) The recovery of the red blood cell ChE activity inhibited to the same degree takes a much longer time

after DFP, the curve showing a flat, almost horizontal portion for the first 1-2 weeks (7). All these findings indicate very strongly that the modes of action of HETP and DFP are not identical, as the dialysis experiments seem to point out. DFP apparently forms a completely irreversible complex with the cholinesterase so that regeneration of the enzyme is necessary before recovery can take place. HETP, on the other hand, appears to form with the enzyme a less stable complex, permitting a certain fraction of the hound enzyme to be released from its combination with the drug. The amount of this reversible fraction, although obviously quite small, is still significant enough to raise within a short time the level of ChE activity above the critical point at which the different signs develop.

It is further evident that the blood cholinesterase is not a true measure of the effect of HETP. This fact has been observed also with DFP (8, 6). The symptomatology, while showing some relationship to the initial depression of the ChE activity, is entirely independent of the further behavior of the blood enzyme. One hour after injection, when the effects of the drug were disappearing, there was still no recovery observed in the ChE activity of either the red cells or the

TABLE IV
Mouse brain ChE recovery after inhibition by HETP

GROUP	HETP	BRAIN ChE ACTIVITY*	REMARKS
	<i>mgm./kgm.</i>	<i>per cent</i>	
I	3.0	67	Sacrificed 15 minutes after injection
II	3.0	81	Sacrificed 60 minutes after injection
III	3.0	74	Sacrificed 120 minutes after injection

* Average of ten brains.

serum. Yet, it is difficult to conceive of symptomatic recovery without enzymatic recovery. It would appear, therefore, that either the body has special reserve stores of the enzyme (in which case HETP should not have been any different from DFP), or tissue cholinesterase forms a more labile combination with HETP than blood cholinesterase. The studies on brain cholinesterase were undertaken to demonstrate this latter possibility. Experiments on both dogs and mice gave suggestive evidence of a rapid return of brain enzymatic function coincident with recovery from the visible effects of the drug. No such enzymatic reactivation was detected with the larger doses of HETP, but this may be explained on a purely physico-chemical basis. After a state of equilibrium has been attained in the body, some of the drug probably remains in the extracellular fluid in an unbound state. When the brain is homogenized preparatory to the manometric determination, this extracellular portion, which *in vivo* produced no effects, now inactivates some more of the enzymes liberated from the ruptured cells and in this way gives rise to false low values.

The objection may be raised that this same mechanism may be the cause for the apparent recovery of the brain enzyme activity; that the low values obtained

when the animal was sacrificed a few minutes after the injection, during the height of the effects, might be due to such an *in vitro* inactivation, and the apparent increase in brain activity after 2-3 hours might be the result of a rapid destruction of HETP in the body so that by that time there was no longer any active HETP remaining in the extracellular fluid. A number of trial experiments were undertaken to prove or disprove this postulate but so far have met with no success. However, the following observation offers some indirect evidence that HETP is not destroyed within the time limits of our experiments: Dogs injected with 2.0 and 5.0 mgm. per kgm. of HETP did not show any recovery of brain ChE activity after 3-4 hours. If HETP were destroyed before this time, one should expect to find an increase in the activity of these samples over those taken a few minutes after administration of the drug. The final answer awaits the study on the fate and rate of destruction of HETP in the body.

SUMMARY

The pharmacologic actions and toxicity of HETP were studied in rabbits, mice and in intact and anesthetized dogs.

HETP produces muscarinic and nicotinic effects of relatively brief duration. That the action is more lasting than these brief observable signs would suggest is shown by the cumulative effects of repeated injections.

The lethal action of toxic doses of HETP in unprotected animals is exerted within 15-30 minutes by intravenous injection, and within 1-2 hours by the intramuscular route. Animals that survive these periods eventually recover.

Death is due to respiratory failure from marked bronchoconstriction and increased bronchial secretion. After atropinization, the respiratory failure results probably from direct central depression or the curariform action of acetylcholine.

Atropine is an effective antagonist of the muscarinic effects of HETP and offers protection against approximately three lethal doses of the drug.

HETP has a potent anticholinesterase action which is responsible for most of the effects of the drug.

In vitro, the inhibition of cholinesterase appears to be mainly irreversible, although the *in vivo* evidence indicates that there is a fairly significant labile component which allows the liberation of sufficient cholinesterase to enable the animal to recover within a short time. HETP, therefore, occupies a position midway between DFP and prostigmine.

The differences between the actions of HETP and DFP were discussed.

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THE EFFECT OF VARIOUS ANESTHETICS AND DECEREBRATION ON THE CO₂ STIMULATING ACTION ON RESPIRATION IN CATS

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It is well known that most non-volatile general anesthetics in dosages sufficiently large to produce surgical anesthesia depress respiration and respiratory reflexes. There have been, however, no data available to estimate quantitatively the degree of depression. The difficulty has been that there is no simple, yet reliable means of recording the respiration quantitatively in unanesthetized animals. Recently, Hemingway (1) has developed a method, which enables one to measure the pulmonary ventilation. With some modification, it was found that the recording of the pulmonary ventilation could be made automatically. Thus, it is possible to determine quantitatively the effect of different anesthetics and decerebration on the stimulating action of CO₂ on respiration.

METHODS To measure the respiratory volume, a body plethysmograph method of the Hemingway type (1) was used (fig 1). An important modification has been made so that the volume of respired gases were periodically removed and automatically recorded. In brief, a normal cat was placed in a closed plethysmograph with head protruding through a seal at the neck. Low inertia valves permitted air to pass through the plethysmograph during respiration. The outlet valve discharged into a 20 liter closed tank, which served as a pneumatic cushion. To the outlet tube from the tank there was connected a small aluminum spirometer and a recording wet test gas meter. Slight suction was constantly applied through the gas meter, but the meter would not register any flow until the opening of a side tube between the meter and suction was blocked by a small metal piece used for balancing the weight of the spirometer. Thus, during each inspiration, the air was expelled from the plethysmograph into the tank. The increase in volume was indicated on the spirometer, which was elevated and thereby lowered the metal piece which blocked the side opening. The suction became effective to remove the added volume of air (equivalent to tidal air) and to return the spirometer to the base line. Consequently, the side tube became open again and suction through the gas meter automatically ceased.

Respiratory rates were obtained by a small tambour connected directly to the plethysmograph. A kymograph record of the respiratory rate, gas meter readings and minute signal markings is shown in figure 2. The amplitude of such respiratory rate recordings is not linearly related to the volume of tidal air. The latter can be computed from the minute volume and the respiratory rate.

For administering the respiratory gases, a constant stream of air was delivered to the cat through a large funnel in front of its head. The volume was at least 20 times the normal respiratory minute volume. When this large volume of air was mixed with the expired air, the resulting CO₂ content was not materially increased so as to influence the respiratory

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movement. When air with increased CO_2 content was desired, a definite quantity of CO_2 was added to the air stream through the same funnel. Since the percentage of CO_2 in these experiments did not exceed 8 per cent of the total gaseous volume, the reduction in the O_2 content of the inspired air was not physiologically important. In practice, we used a constant volume of air, roughly 10 liters per minute, delivered through a capillary tube and measured by a constant pressure with a water manometer in parallel circuit. The variable quantity of CO_2 was also obtained in a similar manner, using variable pressures for different percentages of CO_2 . Once the relation of the percentages of CO_2 content as

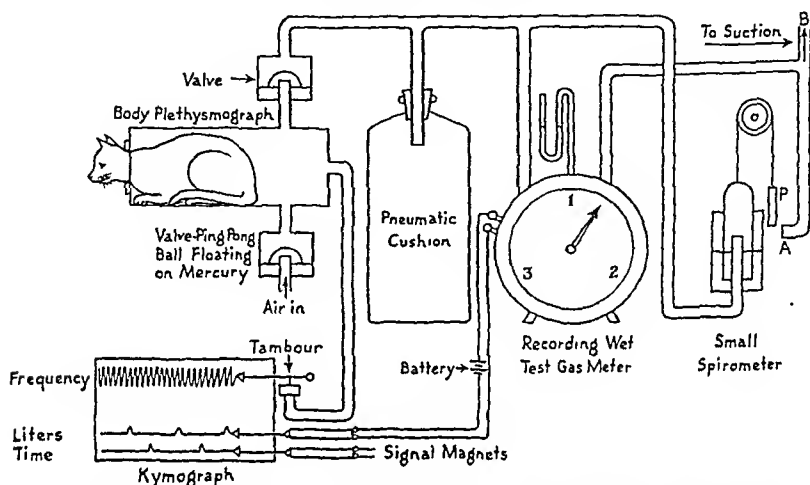


FIG. 1. A DIAGRAMATIC SCHEME OF THE APPARATUS FOR AUTOMATIC QUANTITATIVE RECORDING OF RESPIRATION



FIG. 2. KYMOGRAPH RECORDS OBTAINED IN EXPERIMENT 11.

Upper records, unanesthetized normal cat; lower records, the same cat after 75 mg. of intravenous chloralose per kg. of body weight. Top, respiratory rate, middle, pulmonary ventilation (before anesthesia, in liters; after anesthesia, in 500 cc.); bottom, time in minutes.

estimated by the Haldane apparatus and the corresponding pressures is established, the desired level of CO_2 may be obtained by selecting the correct pressure.

For a standard series, the cat was given air for about 15 minutes or until a fairly constant level of pulmonary ventilation was established. Then air with increased CO_2 was given in steps of 2 per cent increase up to 8 per cent. The time required for the animal to reach a constant level of pulmonary ventilation is different for different cats, and in general it takes relatively less time in cases of higher CO_2 content. The whole series took 30 to 40 minutes. In case the series was repeated, a resting period of approximately one hour was allowed.

Midbrain decerebration was performed on cats under ether anesthesia, and with both

carotid arteries tied (2) Animals with excessive hemorrhage as a result of the operation were discarded. About 30 minutes after injection of the anesthetic or decerebration, the cat was returned to the plethysmograph and control ventilation was again recorded. No increased CO₂ was given until both the rate and minute volume remained fairly constant for 10 to 15 min

RESULTS. Most unanesthetized normal cats showed fairly regular respiration after a few minutes in the plethysmograph. Only a few animals continued to struggle, showed irregular respiratory movements, and were discarded as unsuitable for this type of experiment. A total of 20 experiments were successfully carried out on 19 cats¹.

TABLE 1

Repeated determinations of the respiratory response on normal animals to increased percentage of CO₂ in the inspired air

EX- PERI- MENT NUM BER	BODY WT	CONDITION		0% CO ₂	2% CO ₂	4% CO ₂	6% CO ₂	8% CO ₂
1	1.9	normal	pul vent in cc/min resp rate/min	449(1.00) 18	532(1.55) 42	1955(4.36) 49	2318(7.39) 49	3821(8.51) 49
		normal	pul vent in cc/min resp rate/min	353(1.00) 24	625(1.76) 32	1384(3.92) 40	2515(7.72) 42	3000(8.50) 51
2	1.7	normal	pul vent in cc/min resp rate/min	293(1.00) 25	453(1.65) 23	870(2.97) 25	1756(6.00) 37	2539(8.73) 60
		normal	pul vent in cc/min resp rate/min	333(1.00) 20	576(1.74) 19	1132(3.40) 31	2110(6.34) 32	2904(8.72) 46
3	3.1	normal	pul vent in cc/min resp rate/min	674(1.00) 50	1071(1.59) 53	1727(2.56) 56	2615(3.85) 45	3637(5.44) 48
		normal	pul vent in cc/min resp rate/min	509(1.00) 24	705(1.41) 25	1255(2.53) 29	1944(3.89) 29	2781(5.56) 30
4	2.2	normal	pul vent in cc/min resp rate/min	445(1.00) 34	640(1.66) 34	1429(3.21) 44	2500(5.62) 47	3850(8.65) 53
		normal	pul vent in cc/min resp rate/min	350(1.00) 36	559(1.65) 35	1241(3.55) 44	2176(6.22) 54	2781(7.95) 56

The control series and its repeatability. The unanesthetized normal cat has a pulmonary ventilation varying from 187 to 674 cc. per min., which is only roughly correlated with the body weight of the animal. The average volume was 383 cc. per min. The respiratory rate varied from 15 to 57 per min. with an average of 31 per min. in the normal controls. The maximal pulmonary ventilation on exposure to 8 per cent of CO₂ in air was 4.2 liters per min. and as much as 10.7 times the control, with an average of 2.9 liters per min. or 7.8 times the control.

¹ Experiments 4 and 11 were carried out on the same animals on the same day. Therefore, for computation of the average values in the control series of experiments, the control values in experiment 11 were not included.

The maximal increase in respiratory rate was 2.7 times the control with an average of only 1.3 times. This indicates that following exposure to air with 8 per cent of CO₂ the animal showed an increase both in depth and in rate of respiration. However, the increased pulmonary ventilation was largely due to an increase in the former. Indeed, in several animals in which the control rate was high, only a slight increase, no change, or even a decrease in the respiratory rate on exposure to increased CO₂ was observed, yet the minute volume was higher in every case.

TABLE 2

Effect of nembutal anesthesia on the respiratory response to increased percentage of CO₂ in the inspired air

EX- PERI- MENT NUM- BER	BODY WT.	CONDITION		0% CO ₂	2% CO ₂	4% CO ₂	6% CO ₂	8% CO ₂
5	2.7	normal	pul. vent. in cc./min. resp. rate/min.	618(1.00) 32	1112(1.90) 30	1631(2.98) 30	2942(4.76) 35	3655(5.93) 35
		nembutal 35 mg./kg.	pul. vent. in cc./min. resp. rate/min.	293(1.00) 18	397(1.35) 19	536(1.83) 19	680(3.00) 20	1128(3.55) 22
6	2.7	normal	pul. vent. in cc./min. resp. rate/min.	375(1.00) 15	566(1.51) 16	889(2.37) 20	1608(4.29) 22	2822(7.53) 26
		nembutal 35 mg./kg.	pul. vent. in cc./min. resp. rate/min.	104(1.00) 12	131(1.26) 12	191(1.84) 13	311(3.00) 14	470(4.52) 15
7	2.6	normal	pul. vent. in cc./min. resp. rate/min.	422(1.00) 22	621(1.47) 24	1422(3.37) 27	3084(7.26) 45	4216(9.99) 40
		nembutal 35 mg./kg.	pul. vent. in cc./min. resp. rate/min.	222(1.00) 19	364(1.64) 20	558(2.56) 22	939(4.23) 24	1259(5.67) 28
8	2.2	normal	pul. vent. in cc./min. resp. rate/min.	546(1.00) 51	838(1.53) 49	1371(2.51) 35	2479(4.54) 40	3888(7.12) 43
		nembutal 35 mg./kg.	pul. vent. in cc./min. resp. rate/min.	379(1.00) 24	537(1.42) 27	725(1.91) 25	1038(2.74) 26	1289(3.40) 25

It is essential to establish that after undergoing a complete series of experiments of exposure to a graded increase of CO₂, animals are able to show identical responses following a repetition of the same procedures. Although both the respiratory rate and pulmonary ventilation during the control period had changed⁴ (table 1), the increased pulmonary ventilation in response to increased CO₂ in the inspired air expressed in multiples of the control was not altered during repeated trials. Therefore, for the evaluation of the effect of different procedures on the respiratory response to CO₂, the ratio of increased ventilation to the control minute volume was used.

Nembutal. In 4 cats in which 35 mgm. of nembutal (pentobarbital sodium)

⁴ A great part of the variations could be eliminated through training of the animal.

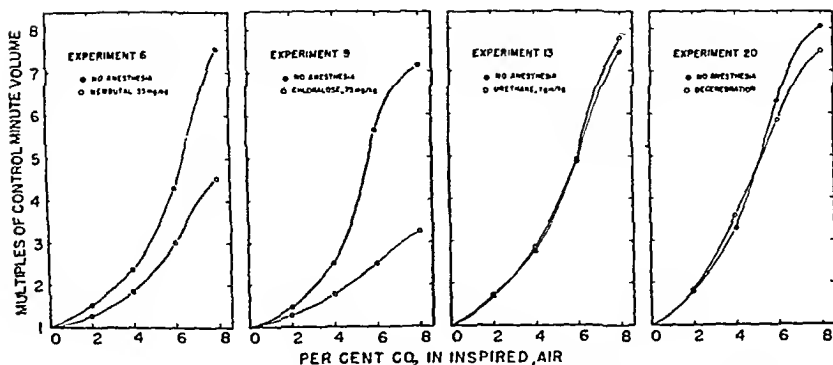


FIG. 3. COMPARISON OF THE CO₂ STIMULATING ACTION ON RESPIRATION IN CATS BEFORE AND AFTER INJECTION OF NEMBUTAL, CHLORALOSE, URETHANE OR DECEREBRATION

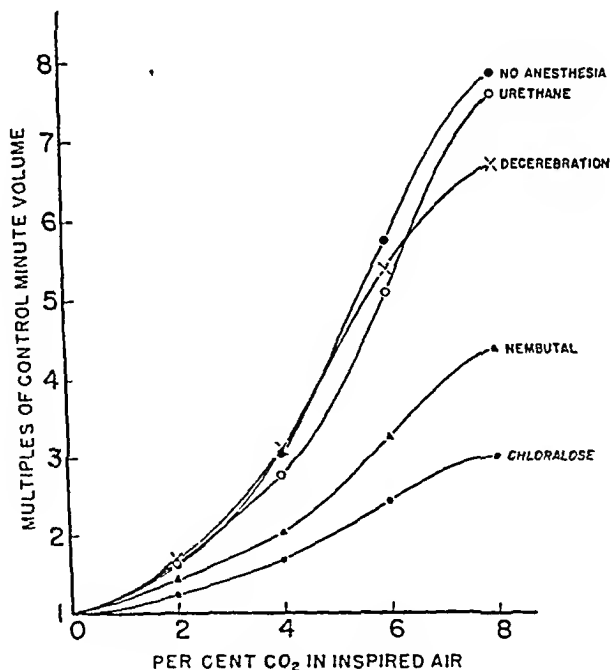


FIG. 4. EFFECT OF VARIOUS ANESTHETICS (NEMBUTAL, CHLORALOSE, AND URETHANE) AND MIDBRAIN DECEREBRATION ON THE CO₂ STIMULATING ACTION ON RESPIRATION

Control, 19 experiments; nembutal, 4 experiments; chloralose, 4 experiments; urethane, 4 experiments; and decerebration, 4 experiments.

per kgm. of body weight were given intraperitoneally, reduction in rate of respiration and pulmonary ventilation during the control period was observed (table 2). The control rate of respiration in the animal under nembutal was reduced to roughly sixty per cent of that found in the same group of animals before injection of nembutal. The percentage of increase in the rate of respiration following inhalation of increased CO_2 , however, was not decreased. On the other hand, the increase in ventilation on exposure to increased CO_2 was greatly reduced in nembutalized animals (figs. 3 and 4). Thus, the reduced sensitivity due to nembutal as judged by the altered pulmonary ventilation is to be ac-

TABLE 3

Effect of chloralose anesthesia on the respiratory response to increased percentage of CO_2 in the inspired air

EX- PERI- MENT NUM- BER	BODY WT.	CONDITION		0% CO_2	2% CO_2	4% CO_2	6% CO_2	8% CO_2
	kg.							
9	2.7	normal	pul. vent. in cc./min. resp. rate/min.	519(1.00) 57	752(1.45) 57	1300(2.50) 49	2917(5.62) 42	3705(7.14) 38
		chloralose 75 mg./kg.	pul. vent. in cc./min. resp. rate/min.	257(1.00) 14	329(1.28) 14	451(1.75) 15	640(2.49) 17	832(3.24) 17
10	2.9	normal	pul. vent. in cc./min. resp. rate/min.	287(1.00) 21	431(1.50) 23	775(2.70) 26	1735(6.05) 28	2330(8.12) 33
		chloralose 75 mg./kg.	pul. vent. in cc./min. resp. rate/min.	108(1.00) 12	126(1.17) 12	198(1.84) 13	294(2.72) 14	370(3.43) 16
10	2.2	normal	pul. vent. in cc./min. resp. rate/min.	350(1.00) 36	589(1.68) 35	1241(3.55) 44	2176(5.22) 54	2781(7.95) 50
		chloralose 75 mg./kg.	pul. vent. in cc./min. resp. rate/min.	155(1.00) 10	191(1.23) 10	231(1.49) 10	293(1.89) 10	343(2.21) 10
12	2.5	normal	pul. vent. in cc./min. resp. rate/min.	337(1.00) 23	563(1.67) 43	1702(5.05) 50	2858(8.48) 56	2823(10.75) 56
		chloralose 75 mg./kg.	pul. vent. in cc./min. resp. rate/min.	201(1.00) 11	251(1.25) 11	317(1.58) 11	531(2.64) 12	623(3.10) 13

counted for by a correspondingly greater depression in the depth than in the rate of the respiratory movement in response to CO_2 inhalation.

Chloralose. A depression similar to, and perhaps more marked than, that of nembutal on the control respiration as well as on the CO_2 stimulating action on respiration was observed in cats, receiving 75 mgm. of chloralose per kgm. of body weight intravenously (table 3 and fig. 3). The control rate of respiration under chloralose was reduced to one third of that before anesthesia. And the effect of CO_2 stimulating action on ventilation was reduced by chloralose to forty per cent of that obtained in the unanesthetized controls (fig. 4). It is important to note that in these cats the spinal reflexes are markedly exaggerated,

so much so that such animals would require additional medication if surgery were desired.

Urethane. No consistent change in the control respiration was obtained in cats receiving one gram of urethane per kgm. of body weight by the intraperitoneal route (table 4). Neither was there any appreciable difference in the animal's response to increased CO₂ (figs. 3 and 4).

Midbrain decerebration. In 4 cats in which decerebration was carried out under ether anesthesia, there was observed a slight increase in control pulmonary ventilation with no consistent change in the rate of respiration as compared with

TABLE 4

Effect of urethane anesthesia on the respiratory response to increased percentage of CO₂ in the inspired air

EX- PER- IMENT NUM- BER	BODY WT.	CONDITION		0% CO ₂	2% CO ₂	4% CO ₂	6% CO ₂	8% CO ₂
	kg.							
13	2.6	normal	pul. vent. in cc./min. resp. rate/min.	315(1.00) 27	542(1.72) 19	870(2.76) 22	1538(4.88) 22	2352(7.47) 27
		urethane 1 gm./kg.	pul. vent. in cc./min. resp. rate/min.	406(1.00) 27	692(1.70) 25	1158(2.85) 27	2000(4.93) 30	3164(7.79) 33
14	2.4	normal	pul. vent. in cc./min. resp. rate/min.	239(1.00) 22	491(1.70) 27	877(3.03) 27	1692(5.85) 33	2000(6.92) 33
		urethane 1 gm./kg.	pul. vent. in cc./min. resp. rate/min.	123(1.00) 27	185(1.50) 28	336(2.73) 32	655(5.57) 32	844(6.87) 29
15	2.6	normal	pul. vent. in cc./min. resp. rate/min.	346(1.00) 47	540(1.50) 42	793(2.29) 40	1515(4.46) 23	2211(6.39) 32
		urethane 1 gm./kg.	pul. vent. in cc./min. resp. rate/min.	303(1.00) 32	446(1.47) 33	641(2.12) 32	1263(4.17) 32	1640(6.07) 33
16	1.8	normal	pul. vent. in cc./min. resp. rate/min.	187(1.00) 18	292(1.66) 19	547(2.93) 22	1129(6.04) 24	2000(10.70) 33
		urethane 1 gm./kg.	pul. vent. in cc./min. resp. rate/min.	159(1.00) 26	305(1.91) 26	529(3.33) 26	888(5.59) 27	1510(9.50) 32

the same group of animals before decerebration (table 5). Following inhalation of air with increased CO₂, the animals showed a comparable increase in the rate of respiration and in pulmonary ventilation. However, when the concentration of CO₂ in the inspired air reached 8 per cent, the increase in ventilation in the decerebrate animals was slightly reduced as compared with the unanesthetized animals (figs. 3 and 4).

DISCUSSION. In unanesthetized normal cats, the pulmonary ventilation is doubled if the CO₂ content in the inspired air is increased to about 2.5 per cent, and tripled if it is raised to approximately 4 per cent. Neither the control ventilation of these animals nor their sensitivity to increased CO₂ in the inspired air is depressed under urethane anesthesia (1 gm. per kgm.) or following de-

cerebration. Though previously no quantitative comparison has been made in these respect, investigators have already found these procedures useful in research in the field of respiratory physiology. For instance, Magoun, et al. (3) has reported that polypneic panting could be demonstrated by application of local heating to the hypothalamus in cats under urethane, but not under other anesthetics. Schmidt (4) has used decerebrate preparations as controls in his study of the influence of narcotic drugs on the sensitivity of the chemoreceptors.

It has been known that nembutal and barbiturates in general depress respiration and respiratory reflexes. Our quantitative data fully support this conten-

TABLE 5

Effect of midbrain decerebration on the respiratory response to increased percentage of CO₂ in the inspired air

EX- PERI- MENT NUM- BER	BODY WT.	CONDITION		0% CO ₂	2% CO ₂	4% CO ₂	6% CO ₂	8% CO ₂
	kg.							
17	1.9	normal	pul. vent. in cc./min. resp. rate/min.	362(1.00) 27	530(1.46) 28	1240(3.43) 33	2173(6.00) 32	2912(8.04) 39
		decere- brated	pul. vent. in cc./min. resp. rate/min.	443(1.00) 30	740(1.67) 38	1325(2.99) 43	2418(5.46) 56	3000(6.77) 60
18	2.1	normal	pul. vent. in cc./min. resp. rate/min.	275(1.00) 30	439(1.60) 33	757(2.75) 33	1716(6.24) 43	2043(7.43) 49
		decere- brated	pul. vent. in cc./min. resp. rate/min.	302(1.00) 23	513(1.70) 26	943(3.12) 30	1600(5.96) 37	2250(7.35) 47
19	1.7	normal	pul. vent. in cc./min. resp. rate/min.	225(1.00) 45	363(1.61) 40	530(2.36) 26	1057(4.70) 28	1395(6.20) 29
		decere- brated	pul. vent. in cc./min. resp. rate/min.	353(1.00) 23	625(1.61) 24	1048(2.70) 30	1640(4.23) 31	2010(6.16) 34
20	2.1	normal	pul. vent. in cc./min. resp. rate/min.	306(1.00) 30	551(1.80) 26	1006(3.29) 33	1925(6.29) 40	2460(8.04) 44
		decere- brated	pul. vent. in cc./min. resp. rate/min.	346(1.00) 31	631(1.83) 33	1242(3.59) 40	2014(5.82) 38	2578(7.45) 28

tion. On the other hand, chloralose, because of its unusual property in facilitating spinal reflexes, is believed by many to be not as markedly depressant to respiration as the barbiturates, yet our data clearly demonstrate that under chloralose both the control respiration and the response to increased CO₂ in the inspired air are depressed to a very great extent (fig. 2). In fact, Schmidt, who believes that chloralose increases the sensitivity of the carotid body receptors to injection of NaCN (4), showed that in animals under chloralose, the response to inhalation of CO₂ may be practically abolished (4, 5, 6). This led Schmidt and his colleagues to believe that under the influence of chloralose the balance of factors in respiratory control is shifted away from the central and toward the reflex regulation (7, 8). In our experiments, chloralose given in dosages as

small as 75 mg. per kg. of body weight, is shown to have a marked depression on the respiration. This effect is similar to that observed in case of nembutal (35 mg. per kg., I. P.). We have obtained no evidence indicating that the chemoreceptive reflex mechanism under chloralose is different from that under nembutal. It is important to stress here that in an animal in which spinal reflexes are exaggerated (such as under chloralose), reflex excitability in the control respiration may not be necessarily increased.

SUMMARY AND CONCLUSIONS

A method of automatic recording of the pulmonary ventilation of unanesthetized animals (a modification of the Hemingway's body plethysmograph method) was described.

It was found that the control ventilation and the sensitivity of the respiratory system to increased CO₂ are not appreciably altered by urethane (1 gm. per kg., I. P.) or by midbrain decerebration. On the other hand, nembutal (35 mg. per kg., I. P.) or chloralose (75 mg. per kg., I. V.) markedly depresses the control respiration as well as respiratory response to increased CO₂ content in the inspired air.

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INVESTIGATION OF SOME NEWER SULFONAMIDES AS INTESTINAL CHEMOTHERAPEUTIC AGENTS

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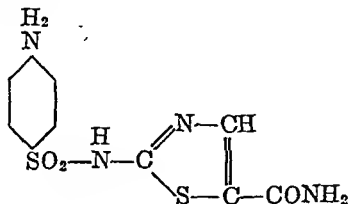
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Investigation of a number of N⁴-dicarboxylic acid-substituted sulfonamides (1, 2, 3, 4) has shown that certain of these, while not possessing antibacterial activity in vitro, do exhibit marked action against organisms of the intestinal flora when tested in vivo. Two of the most active of the derivatives studied were succinylsulfathiazole (sulfasuxidine) and phthalylsulfathiazole (sulfathalidine), which have now become well established as intestinal chemotherapeutic agents.

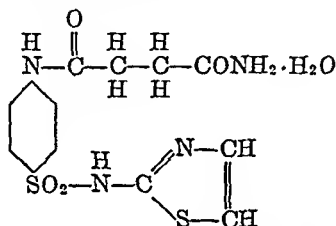
In addition to the N⁴-acylated derivatives, sulfonamides have been synthesized (5, 6, 7, 8, 9, 10) containing carboxy, carbomethoxy, carbamyl, "tert."-amino-alkyl, hydroxymethyl or alkylmercaptomethyl groups in the heterocyclic nucleus. Some of these (2-sulfanilamido-4-carboxythiazole, 2-sulfanilamido-5-carboxythiazole and 2-sulfanilamido-4,5-dicarboxythiazole) have also indicated promise as effective intestinal antiseptics (11, 12, 13, 14).

In an extended study of carboxy derivatives of sulfathiazole, Sprague, Lincoln and Ziegler (8) found that stability was greatly influenced by the position of the group. A carboxy group in the 5-position of the thiazole ring or a carboxymethyl group in the 4-position was quite labile, while a 4-carboxy or 5-carboxymethyl group remained relatively stable. Experimental evidence has shown that such carboxylation results, in general, in the same types of pharmacological and bacteriological response as have been observed with the N⁴-substituted sulfonamides, i.e., diminished absorption from the gastro-intestinal tract, lack of toxicity, reduced antibacterial action in vitro but exhibiting more or less marked activity in vivo. Regarding this last property, Ellingson, Henry and McDonald (9) reported recently that while introduction of the carboxy and carbomethoxy groups in the 3-position of sulfapyrazine either greatly reduced or destroyed antibacterial activity in vitro, the presence of the carbamyl group had little or no influence.

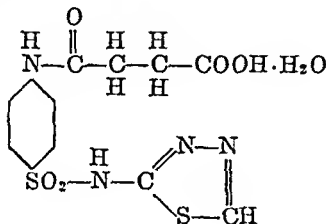
In respect to the investigation of chemotherapeutic agents for intestinal use, the present report consists of preliminary data on three new sulfonamides which may offer some additional information on the relation of chemical structure to possible therapeutic effect.

COMPOUNDS.¹ 1. 2-Sulfanilamido-5-carbamylthiazole:

(M.W., 298.3; M.P., 273°C. with decomposition; heat stable; soluble in dilute NaOH and HCl)

2. 2-(N⁴-Succinamylsulfanilamido)-thiazole:

(M.W., 372.4; M.P., 214°C. with decomposition; heat labile, eliminating NH₃ in warm NaOH; soluble in NaOH, NH₄OH, hot 30% ethanol, and in less than 2.0 mg. per 100 cc. of distilled water at room temperature)

3. 2-(N⁴-Succinylsulfanilamido)-1,3,4-thiadiazole:

(M.W., 374.4; M.P., 221-223°C. with decomposition; heat labile (Table I); soluble in dilute NaHCO₃ solution)

EXPERIMENTAL. Absorption. Blood concentrations were determined in mice (CFW) following oral administration of single doses of drug at 0.5 and 1.0 gm. per kilogram, respectively. Animals weighing 20 gm. each were used throughout and the drug suspended in 10 per cent gum acacia in concentrations yielding the required dose in a volume of 0.5 cc. Food was withheld for 16 hours prior to dosing and for 8 hours after. Blood levels on pooled samples (10 mice) were run according to the method of Bratton and Marshall (16). For

¹ The compounds were synthesized in this laboratory; the details of which will be described elsewhere (15).

complete hydrolysis with succinylsulfathiadiazole and succinamylsulfathiazole it was necessary, as with sulfasuxidine, to increase the concentration of HCl to 1N and the heating time to 2 hours. With sulfathalidine, hydrolysis was also carried out for 2 hours, but in the presence of only 0.2 N HCl. Because of the rapidity with which sulfathalidine is broken down in acid solution, the time interval between the addition of trichloroacetic acid and sodium nitrite was reduced to a minimum and kept constant for all free determinations.

Blood concentrations with succinylsulfathiadiazole were low and comparable to those obtained with sulfasuxidine and sulfathalidine (Table II). Succinamylsulfathiazole levels were negligible. Lower peak concentrations resulted with sulfacarbamylthiazole than were produced by the corresponding carboxylic

TABLE I

Free Diazotizable substance in sulfonamide solutions before and after autoclaving

DRUG	CONCENTRATION OF DRUG BY WEIGHT	TREATMENT	DIAZOTIZABLE SUBSTANCE*	
			Concentration	Per cent of total weight
	mg. %		mg. %	
Succinylsulfathiadiazole	80	Unheated	0.42	0.53
		5 min. heat	1.08	1.35
		20 min. heat	1.85	2.31
Succinamylsulfathiazole	9	Unheated	0.03	0.33
		5 min. heat	1.69	18.78
		20 min. heat	2.50	27.78
Sulfasuxidine	64	Unheated	0.19	0.30
		5 min. heat	0.40	0.63
		20 min. heat	0.64	1.00
Sulfathalidine	64	Unheated	0.62	0.97
		5 min. heat	5.15	8.05
		20 min. heat	9.80	15.31

* Calculated as free sulfathiadiazole and sulfathiazole, respectively.

acid derivative of sulfathiazole. Administration of the N⁴-derivatives to mice at the 2 dose levels employed did not result in any significant difference in blood concentrations. The effect of introducing the succinyl radical at the N⁴-position of sulfathiadiazole was clearly shown by the marked difference in blood concentrations when compared in this respect with sulfathiadiazole.

Acute toxicity. Drugs suspended in 10 per cent gum acacia were administered orally to mice (CFW) weighing 20 grams. Blood levels were determined from pooled samples obtained 2 hours after dosing. Results are summarized in Table III. Succinamylsulfathiazole and sulfacarbamylthiazole failed to kill mice in doses as high as 20 gm. per kilogram, comparing equally in this respect with sulfaguanidine, sulfasuxidine and sulfathalidine. The LD₅₀ of sulfacarboxythiazole was found to be 10 gm. per kilogram, while succinylsulfathiadiazole proved fatal to 50 per cent of animals at 15 gm. per kilogram.

In vitro activity. Because of the chemical structure of succinylsulfathiadiazole and succinamylsulfathiadiazole no activity *in vitro* was expected. Using a procedure previously described (17), it became apparent that antibacterial action shown by various samples of the compounds was due to slight contamination, presumably sulfathiadiazole and sulfathiazole, respectively. The amount of free diazotizable substance could be increased, along with a corresponding increase in activity, by prolonged autoclaving of the N^4 -derivatives in solution (Table I).

While sulfacarboxythiazole possesses some bacteriostatic activity *in vitro* per se, a greater action due to decomposition is indicated in tests in which heat is

TABLE II

Blood concentrations of sulfonamides in mice following single oral dose of 1.0 gm. per kilogram

DRUG	DRUG CONCENTRATION IN MG. PER 100 CC. OF BLOOD									
	1 hr.		2 hr.		4 hr.		8 hr.		24 hr.	
	Free	Combined*	Free	Combined*	Free	Combined*	Free	Combined*	Free	Combined*
Sulfathiazole	17.7	0.6	21.2	0.9	14.1	0.3	5.5	0.1	0.2	0.0
Sulfaguanidine	3.8	0.5	4.9	1.4	3.4	0.5	3.4	0.4	0.2	0.3
Sulfathiadiazole	14.5	0.4	10.7	0.5	5.1	0.6	1.8	0.7	0.0	0.2
Succinylsulfathiadiazole	0.5	2.7	0.4	2.8	0.2	2.4	0.2	2.4	0.0	0.0
Sulfacarboxythiazole	2.8	0.0	1.9	0.1	1.3	0.2	1.1	0.3	0.2	0.0
Sulfacarbamylthiazole	0.8	0.0	1.2	0.0	1.0	0.0	0.8	0.0	0.0	0.0
Sulfasuxidine	0.7	1.1	0.6	0.7	0.3	0.5	0.2	0.6	0.0	0.0
Sulfathalidine . . .	0.3	0.4	0.2	0.4	0.2	0.1	0.2	0.1	0.0	0.0
Succinamylsulfathiazole	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0

* Combined drug for N^4 -derivatives expressed as succinylsulfathiadiazole, sulfasuxidine, sulfathalidine and succinamylsulfathiazole, respectively

used for sterilization of solutions. The carbamyl derivative of sulfathiazole, however, is quite stable to heat and definite activity equivalent to that of sulfanilamide and sulfaguanidine was shown against members of the colon-typhoid-dysentery group (Table IV)

In vivo activity: In an attempt to evaluate the probable effect of the compounds in enteric infections, antibacterial activity *in vivo* was first determined in mice following the method described by White (19). Mice (CFCW) were set up into groups of 5 animals each, all groups being comparable in respect to weight and average coli counts. Drug was administered in a 1 per cent drug-diet for 7 days, at the end of which time coli counts were again estimated. Eijkman lactose broth containing 50 mg. of para-aminobenzoic acid per 100 cc., dispensed in fermentation tubes, was used as the test medium, and incubation carried out in a constant temperature water-bath at 46°C. All positive tubes were streaked on eosin methylene blue agar (Levine) for confirmation of coli. Blood concentrations of drug were determined on the second and fifth days of test and fecal concentrations on the seventh.

Results of the anti-coli test in mice, including those obtained with other known sulfonamides, are given in Table V. Sulfathiadiazole, sulfathiazole and sulfacarboxythiazole in the order named were the most active of the drugs studied. Sulfasuxidine, sulfathalidine and succinamylsulfathiazole were about equally the least active, while sulfacarbamylthiazole and succinylsulfathiadiazole occupied

TABLE III
Acute oral toxicities of sulfonamides in mice

DRUG	DOSE	NUMBER DEAD/TOTAL	BLOOD CONCENTRATION 2 HOURS AFTER DOSE	
			Free	Combined*
	<i>gm. per kg.</i>		<i>mg. per 100 cc.</i>	
Sulfacarbamylthiazole	1.0	0/10	1.2	0.0
	10.0	0/10	1.9	0.0
	20.0	0/10	2.3	0.0
Sulfacarboxythiazole	1.0	0/10	1.9	0.1
	10.0	5/10	5.9	2.0
	12.0	15/20	7.2	2.1
	14.0	10/10	15.7	2.3
Succinylsulfathiadiazole	1.0	0/10	0.4	2.8
	5.0	0/8	0.4	5.8
	10.0	3/10	1.1	10.6
	15.0	5/10	1.3	11.8
	20.0	10/10	6.1	15.2
Succinamylsulfathiazole	1.0	0/10	0.0	0.0
	10.0	0/10	0.3	0.0
	20.0	0/20	0.3	0.4
Sulfasuxidine	1.0	0/10	0.6	0.7
	20.0	0/10	2.5	3.1
Sulfathalidine	1.0	0/10	0.2	0.5
	20.0	0/10	1.5	1.1
Sulfaguanidine	1.0	0/10	4.9	1.4
	20.0	0/10	3.4	1.4

* Combined drug for N⁴-derivatives expressed as succinylsulfathiadiazole, succinamylsulfathiazole, sulfasuxidine and sulfathalidine, respectively.

more or less intermediate positions in effectiveness. Blood levels were highest in sulfathiazole-treated mice, lowest in animals on drug-diets containing the N⁴-substituted derivatives.

Subsequent to the assay of anti-coli activity in mice a similar study was carried out in dogs. The animals were maintained solely on horse meat fortified with cod liver oil² for a

² 40 grams of horse meat per kilogram of body weight and 2 cc. of cod liver oil daily.

TABLE IV

Activity in vitro of sulfonamides against organisms of the colon-typhoid-dysentery group

DRUG	LOWEST CONCENTRATION OF DRUG (MG. PER 100 CC.) PREVENTING VISIBLE GROWTH OF FOLLOWING ORGANISMS AFTER 72 HOURS INCUBATION						
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>Sal. enteritidis</i>	<i>Sal. pullorum</i>	<i>Sh. dysenteriae</i>	<i>Sh. gallinarum</i>	<i>V. cholerae</i>
Sulfanilamide.....	5.0	5.0	>80.0	80.0	5.0	80.0	10.0
Sulfaguanidine.....	5.0	10.0	80.0	80.0	1.25	>80.0	10.0
Sulfathiazole.....	0.08	0.16	2.5	1.25	0.04	2.5	0.03
Sulfathiadiazole.....	1.25			5.0		20.0	
Sulfacarboxythiazole (autoclaved 5 min.).....	0.63	2.5	40.0		0.31		1.25
Sulfacarboxythiazole (Seitz-filtered)...	5.0	20.0	>80.0	40.0	20.0	>80.0	40.0
Sulfacarbamylthiazole (autoclaved 5 min.).....	5.0	20.0	>80.0	80.0	5.0	>80.0	10.0
Sulfacarhamylthiazole (Seitz filtered)...	5.0	20.0			5.0		

The asparagine-glucose-salts medium described by Sahyun and associates (18) was used with *E. coli* and *A. aerogenes*; enrichment of the synthetic medium with 0.1 per cent casein hydrolysate served for the remaining enteric strains. Inocula varied between 500 and 700 organisms per cc.

TABLE V

Anti-coli activity of sulfonamides in mice

DRUG*	DAILY INTAKE		DRUG CONCENTRATION				LOGARITHMIC COLI COUNT		
	Drug-diet	Drug	Blood		Feces		Before treatment	After treatment	Reduction
			5th day		7th day				
			Free	Combined†	Free	Combined†			
	gm.	gm. per kg.	mg. per 100 cc.		mg. per 100 gm.				
Sulfathiadinazole.....	4.2	2.5	2.4	0.0	697	0	5.6	0.4	5.2
Sulfathiazole	5.0	2.2	4.4	0.1	252	10	5.2	0.6	4.6
Sulfacarboxythiazole	4.5	2.7	0.6	0.0	1502	7	5.8	1.6	4.2
Sulfacarbamylthiazole .	4.4	2.6	2.1	0.0	1605	7	5.6	2.0	3.6
Succinylsulfathiadiazole .	4.2	2.3	0.4	0.5	59	2070	5.4	2.0	3.4
Succinamylsulfathiazole .	5.1	2.2	0.1	0.4	3.6	1640	4.4	3.4	1.0
Sulfasuxidine... .	5.2	2.2	0.1	0.3	15	2290	4.4	3.6	0.8
Sulfathalidine... .	4.5	2.2	0.2	0.5	148	1950	5.0	4.2	0.8
Control..... .	4.7						4.9	5.1	-0.2

Above figures are average values for 5 mice in each drug group and for 10 controls.

* Each drug incorporated in diet in 1 per cent concentration.

† Combined drug for N⁴-derivatives expressed as succinylsulfathiadiazole, succinamylsulfathiazole, sulfasuxidine and sulfathalidine, respectively.

time sufficient for the establishment of an intestinal flora consistent with such a diet. Each drug was tested on 3 dogs at a dosage of 1.0 gm. per kilogram daily. The drug was incorporated in the meat, and the daily dose administered in 2 equally divided amounts: one in the morning, the second 8 hours later. Treatment was continued for 4 days. Fecal samples for bacteriological analysis were obtained every 24 hours directly from the rectum by means of sterile glass tubing. Samples were placed in sterile 1 ounce bottles, and an initial dilution of 1:10 prepared on the basis that 1.0 gm. of wet feces was equivalent to 1.0 cc. Serial dilutions were prepared in multiples of 10, and appropriate dilutions plated in desoxycholate agar for the enumeration of coli organisms. The medium contained 5.0 mg. of para-aminobenzoic acid per 100 cc., and incubation was at 37°C. for 24 hours. Blood and fecal concentrations of drug were determined daily. Because of the slowness with which fecal suspensions filter through, all samples were clarified (Whatman paper #50) before the addition of trichloroacetic acid to obviate any hydrolysis of the N^4 -derivatives, which would in effect give rise to false readings. As a further precaution, sodium nitrite was always added first, followed immediately by the trichloroacetic acid. Since several passages through paper are often necessary to obtain reasonably clear fecal filtrates, an additional modification of the procedure has been adopted. Thus, for each fecal sample analyzed, excess turbidity was determined in the colorimeter by treating an aliquot of the filtrate in the same manner as the test portion, but omitting the color-producing reagent. Final readings were adjusted accordingly.

As indicated in Table VI, relatively low blood concentrations of the sulfonamides resulted in dogs at the high dosage administered. Greatest levels were produced by sulfacarboxythiazole (1 to 5 mg. per 100 cc. of blood), while no absorption whatever was observed with succinamylsulfathiazole. Results of fecal analyses are summarized in Table VII. The free drug levels found in feces of dogs given the N^4 -derivatives indicated varying degrees of hydrolysis of these compounds in the gastro-intestinal tract. Maximal conversion to free drug occurred after the administration of sulfathalidine, while very little if any breakdown to free component was apparent with succinylsulfathiadiazole.

Two methods of comparison were used in evaluating the antibacterial activity of the sulfonamides in dogs: one based on actual individual coli counts in each group, the second in which counts were expressed in terms of per cent of normal coli numbers. In either case, the order of effectiveness was found to be the same: sulfacarboxythiazole > sulfathalidine > succinylsulfathiadiazole > sulfacarbamylthiazole > succinamylsulfathiazole (fig. 1).

DISCUSSION. Consideration of N^4 -substituted sulfonamides and certain derivatives of sulfathiazole having various functional groups in the thiazole nucleus, reveals several characteristics possessed more or less in common. Greatly reduced absorption from the gastro-intestinal tract and relative lack of toxicity are perhaps the most consistent findings. Of therapeutic interest is the fact that these compounds, while showing either no action in vitro (N^4 -derivatives) or markedly reduced activity (substituted heterocyclic compounds), do exhibit in a number of instances pronounced antibacterial effect in vivo.

Regarding the mode of action of the N^4 -type sulfonamides, it has been generally assumed that the activity of these compounds, particularly sulfasuxidine and sulfathalidine, is due to free drug liberated as a result of hydrolysis in the intestine. Data presented here, however, do not seem to substantiate this

belief. No apparent relationship could be noted between activity of the N⁴-derivatives and fecal concentrations of drug in either mice or dogs. If reduction in the numbers of coli is an expression of the amount of free diazotizable material (sulfathiazole, etc.) present in feces (2, 4, 19, 20), then a greater decrease in count should have resulted in mice receiving sulfathalidine than actually occurred (Table V). The administration of succinylsulfathiadiazole to dogs resulted in little, if any, free drug in feces (Table VII); yet this compound exhibited

TABLE VI

Blood concentrations of sulfonamides in dogs following oral dose of 1.0 gm. per kilogram daily

DRUG	DOG NO.	DRUG CONCENTRATION IN MG. PER 100 CC. OF BLOOD							
		1st day*		2nd Day*		3rd Day*		4th day*	
		Free	Combined†	Free	Combined†	Free	Combined†	Free	Combined†
Sulfacarbamyl-thiazole	1	0.5		0.7				0.7	
	2	0.5		0.9				1.1	
	3	0.6		0.7				0.8	
Sulfacarboxy-thiazole	4	1.1		1.5		2.5		3.5	
	5	2.3		2.3		3.3		4.9	
	6	1.5		0.9		3.3		2.8	
Succinylsulfathiadiazole	7	0.0	0.3	0.1	0.6			0.1	1.2
	8	0.1	0.3	0.2	0.6			0.1	0.6
	9	0.0	0.2	0.0	0.4			0.1	2.1
Succinamylsulfathiadiazole	10	0.0	0.0	0.0	0.0			0.0	0.0
	11	0.0	0.0	0.0	0.0			0.0	0.0
	12	0.0	0.0	0.0	0.0			0.0	0.0
Sulfathalidine	13	0.0	0.0	0.4	0.1	0.6	0.1	0.6	0.0
	14	0.1	0.1	0.4	0.1	0.5	0.1	0.5	0.1
	15	0.1	0.1	1.6	0.1	0.7	0.1	0.5	0.0

* Blood samples obtained 4 hours after morning dose.

† Combined drug expressed as succinylsulfathiadiazole, succinamylsulfathiadiazole and sulfathalidine, respectively.

anti-coli activity almost as great as did sulfathalidine in the same animal. If the above theory were plausible, sulfathalidine should have again been expected to show more activity than was indicated in dogs, on the basis of comparatively higher free concentrations of drug in feces and the fact that sulfathiazole is about 16 times more active than sulfathiadiazole against *E. coli* in vitro (Table IV). Toward the end of the test period in dogs, greatest combined drug concentrations in stools were obtained with the least effective drug, succinamylsulfathiadiazole. On the other hand, the marked action against coli with succinylsulfathiadiazole was maintained with approximately one-third as much combined drug. These observations would thus appear to strengthen the recent conclusion of Poth

and Ross (21) that sulfonamides of the N^4 -substituted type probably owe their activity to some property of the conjugated molecule rather than to any free active component resulting from hydrolysis in the body.

The present investigation offers some pertinent information concerning the relative merits of animal experimentation, and serves to re-emphasize the necessity of using several species of animals in studies of drug activity. Changes in

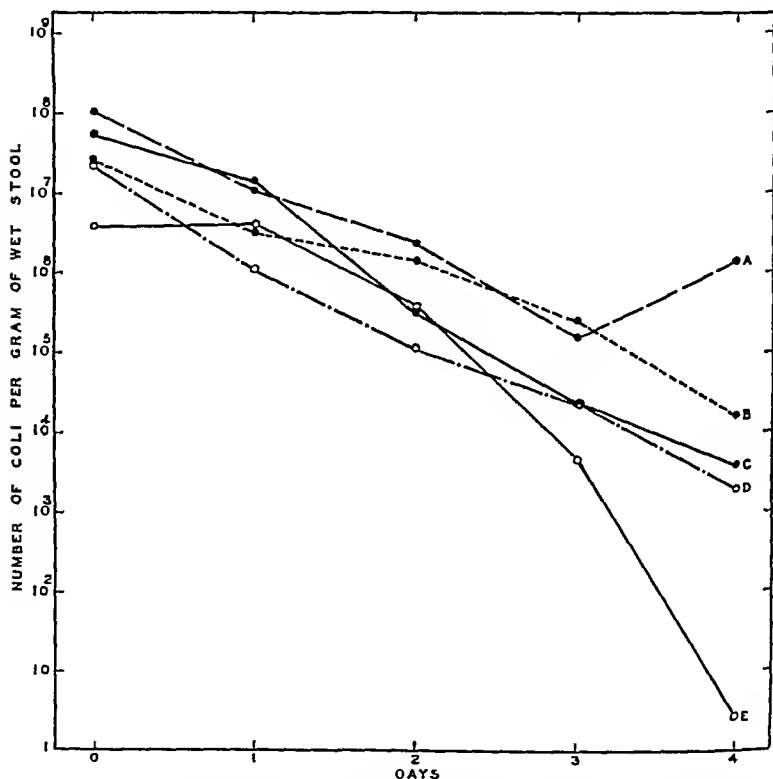


FIG. 1. Comparative anti-coli activities of sulfonamides in dogs. Data are given as averages of the individual coli counts for 3 animals per drug. Respective drugs were administered in a dosage of 1.0 gm. per kilogram daily. A, succinamylsulfathiazole; B, sulfacarbamylthiazole; C, succinylsulfathiadiazole; D, sulfathalidine; E, sulfacarboxythiazole.

the order of effectiveness of the drugs against coli were quite apparent upon comparison of results obtained in mice with those in dogs. For example, sulfacarboxythiazole was the most active of the drugs that were tested respectively in both animals. On the other hand, succinylsulfathiadiazole, which was much more effective than sulfathalidine in mice, appeared slightly less active than the latter when tested in dogs. Insignificant coli reductions with sulfathalidine

and sulfasuxidine are practically always obtained in mice, and the fact that such results are not in agreement with those observed in larger animals and in clinical practice tends to limit the usefulness of the mouse anti-coli test. Although this test offers several obvious advantages, it is nevertheless possible in the screening of sulfonamides to miss one or more possibly good drugs, especially when dealing with N⁴-substituted derivatives.

White and associates (22) believed that sulfathiadiazole offered one of the most promising compounds available for trial in the treatment of bacillary dysentery. Meads and Finland (23), however, found the compound too rapidly

TABLE VII

Fecal concentrations of sulfonamides in dogs following oral dose of 1.0 gm. per kilogram daily

DRUG	DOG NO.	DRUG CONCENTRATION IN GM. PER 100 GM. OF WET FECES							
		24 hr.		48 hr.		72 hr.		96 hr.	
		Free	Com-bined*	Free	Com-bined*	Free	Com-bined*	Free	Com-bined*
Sulfacarbamyl-thiazole	1	2.53		4.25		6.79		1.54	
	2	0.047		0.072		3.07		5.34	
	3	0.98		0.08		17.7		23.8	
Sulfacarboxy-thiazole	4	0.673		3.47		3.93		4.56	
	5	3.67		4.30		4.39		4.64	
	6	1.03		4.41		4.80		4.51	
Succinylsulfathiadiazole	7	0.017	3.38	0.0	3.88	0.005	4.13	0.0	4.01
	8	0.05	2.55	0.003	3.92	0.0	3.38	0.0	4.03
	9	0.012	4.62	0.03	4.39	0.015	3.78	0.0	4.02
Succinamylsulfathiadiazole	10	0.045	1.78	0.065	5.24	0.103	7.43	0.065	15.5
	11	0.037	1.40	0.033		0.105	5.66	0.075	13.7
	12	0.035	0.045	0.025	4.90	0.085	4.62	0.05	13.3
Sulfathalidine	13	0.0	0.0	0.202	7.14	0.247	6.46	0.367	11.1
	14	0.183	6.4	0.227	6.76	0.188	6.89	0.305	8.28
	15	0.002	0.002	0.168	6.49	0.253	10.1	0.256	9.25

* Combined drug expressed as succinylsulfathiadiazole, succinamylsulfathiadiazole and sulfathalidine, respectively.

absorbed in man to be particularly useful in enteric infections. Because of its rapid elimination and high solubility in the urinary pH range, they suggested rather that the drug be tried in urinary tract infections.

Work here has indicated that sulfathiadiazole can be modified through N⁴-substitution of the succinyl radical so that it is only sparingly absorbed, yet soluble in high concentrations in intestinal contents. These two properties, considered desirable criteria for a good intestinal antiseptic, in addition to its marked antibacterial action in vivo make succinylsulfathiadiazole especially worthy of more study.

SUMMARY

Preliminary study of three new sulfonamides as intestinal chemotherapeutic agents is reported: 2-sulfanilamido-5-carbamylthiazole, 2-(N⁴-succinamylsulfanilamido)-thiazole, and 2-(N⁴-succinylsulfanilamido)-1,3,4-thiadiazole.

The presence of the carbamyl group in the 5-position of sulfathiazole greatly reduces absorption from the gastro-intestinal tract and activity in vitro. However, marked anti-coli action was demonstrated in mice and dogs. Sulfacarbamylthiazole is relatively stable and activity is apparently due to the drug per se.

Replacement of the carboxy group of succinylsulfathiazole with the carbamyl group yields a compound considerably more labile than the parent drug. While absorption was slight following oral administration of succinamylsulfathiazole to mice and dogs, the drug showed but little anti-coli activity in vivo.

Substitution of the succinyl radical in the N⁴-position of sulfathiadiazole results in a compound highly active in the intestinal tract of both mice and dogs and only slightly absorbed into the blood. The anti-coli activity of succinylsulfathiadiazole was found to be much greater than that of sulfathalidine in mice and about equal to the latter drug in dogs. Data seem to establish succinylsulfathiadiazole as a potentially useful drug for trial in therapy of enteric infections.

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THE VASOPRESSOR ACTIVITY OF SOME NEW ORALLY ACTIVE SYMPATHOMIMETIC AMINES

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Orally active sympathomimetic amines have been studied by many investigators. This paper describes an investigation of the relative vasopressor efficiency of a small group of orally active phenyl- and cyclohexylisopropylamines.

The general pharmacology of some of these compounds has already been described by Tainter (1), and Lands, Nash, Granger, and Dertinger (2).

The compounds investigated in this series of experiments are 1-(*m*-hydroxyphenyl)-2-aminopropanol HCl, 1-(*p*-hydroxycyclohexyl)-2-amino-propane HBr, with its *N*-methyl homologue and the optical isomers of 1-cyclohexyl-2-methylaminopropane.

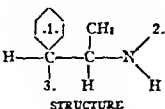
METHODS AND MATERIALS In dogs anesthetized with sodium pentobarbital, aqueous solutions of the compounds under test were injected either intravenously into the exposed femoral vein, or directly into the lumen of an exposed loop of duodenum. Selected unanesthetized dogs, trained to lie quietly with a modified sphygmomanometer cuff fastened about their left thigh, were given aqueous solutions of the drugs by gastric intubation. Systolic blood pressure was determined by palpating the anterior tibial artery. Heart rates were obtained by either palpation or auscultation.

RESULTS. The comparative vasopressor activity of the compounds investigated is shown in tables 1 and 2. Propadrine is included in these tables for purposes of comparison, inasmuch as its pharmacology has been described by Chen, Wu, and Henriksen (3), and by others. It will be noted that compound O-4,2 is the most active at all dose levels where direct comparisons are possible. This was especially noted following intravenous administration. Compound O-4,350 appears to be approximately as active as 'Propadrine' when administered intra-intestinally or intravenously in anesthetized dogs (table 2). Its primary amine homologue, compound O-4,345, shows comparable activity in the anesthetized dog when it is placed within the gut, but it has only about one-third as much activity when given intravenously, or orally in unanesthetized dogs.

With the optical isomers of 1-cyclohexyl-2-methylaminopropane the 1:1:d ratio equals 2:5 (Lands, *et al.* 2). This ratio is quite well borne out in the results obtained by both intra-intestinal and intravenous injections in anesthetized dogs, but the difference is not discernible in data obtained with unanesthetized animals, where the drug is given by intubation. The difference in activity, both in the case of these optical isomers, and between O-4,2 and O-4,350, although readily apparent in the anesthetized animals, becomes much less significant, or disappears altogether in unanesthetized animals given the drug orally. Reasons for these

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TABLE 1
Pressor activity of several orally active vasoconstrictors

COMPOUND	 STRUCTURE			ANESTHETIZED DOGS (DRUG ADMINISTERED INTRA-INTESTINALLY)				UNANESTHETIZED DOGS (DRUG ADMINISTERED PER OS.)				
	1	2	3	Dose	B.P. change	Dur.	No. exp.	Dose	B.P. rise*	Time†	Dur.‡	No. exp.
				mgm / kgm.	mm. Hg	min.		mgm./ kgm.	mm. Hg	min.	min.	
O-4,2 HCl	phenyl m-OH	H	OH	1.0 2.0	+40 +76	99 85	6 2	0.50 1.00	+34 +42	80 57	100 >200	3 6
O-4,350 SO ₄	cyclo- hexyl p-OH	CH ₃	H	1.0 3.0	+27 +34	>73 >34	5 4	1.00	+33	36	>120	7
O-4,345 HBr	cyclo- hexyl p-OH	H	H	3.0	+42	>51	5	3.00	+39	38	>100	6
O-4,1172 HCl (d-isomer)	cyclo- hexyl	CH ₃	H	5.0	+34	>74	6	3.00	+45	47	>130	6
O-4,1171 HCl	(l-isomer of above compound)			3.0	+46	>55	8	3.00	+42	55	>150	5
'Propadrine HCl'	phenyl	H	OH	1.0	+31	67	5					

* Blood pressure rise = maximal difference in normal blood pressure and blood pressure after administration of compound.

† Time = minutes necessary for maximal change in blood pressure.

‡ Duration = time during which blood pressure remained significantly above normal.

TABLE 2
Blood pressure effects and toxicity of some vasopressor amines

COMPOUND	ANESTHETIZED DOGS (DRUG ADMINISTERED INTRAVENOUSLY)				TOXICITY (ALBINO MICE I.P. INJECTIONS)	
	Dose	B.P. change	Dur.	No. exp.	LD ₅₀ dose	No. of animals
	mgm./kgm.	mm. Hg	min.		mgm./kgm.	
O-4,2	0.08	+41	8	6	440	50
O-4,350	0.58	+63	13	9	580	63
O-4,345	0.50	+26	57	7	1000	37
O-4,1172	0.50	+31	10-30	6	75	20
O-4,1171	0.26	+41	8-30	6	73	30
'Propadrine'	0.50	+73	42	8	428	60

differences in response are not readily apparent. From plotted charts (see figures 1 and 2) based on frequent blood pressure determinations made during the first 3 hours following drug administration, difference in the rate of absorption

of the drug suggests itself as an important factor. An examination of the time columns will reveal that all the compounds are long acting in their pressor effects. With the unanesthetized dog only blood pressure rises of more than 15 mm. of mercury were considered significant, and the duration of effect was calculated on that basis.

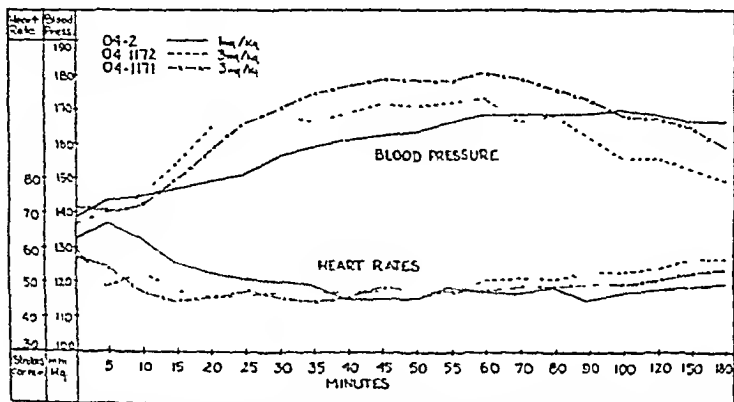


FIG 1. Comparison of the effect on blood pressure and heart rate of 1-(m-hydroxyphenyl)-2-aminopropanol HCl, d-and-l-cyclohexyl-2-methylaminopropane HCl.

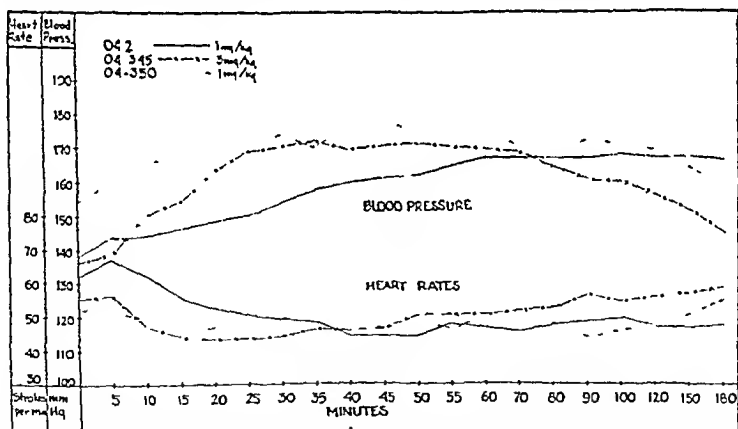


FIG 2 Comparison of the effect on blood pressure and heart rate of 1-(m-hydroxyphenyl)-2-aminopropanol HCl, 1-(p-hydroxycyclohexyl)-2-aminopropane HBr and 1-(p-hydroxycyclohexyl)-2-methylaminopropane sulfate

Toxicity, as shown in table 2, was determined by intraperitoneal injection into albino mice. All animals were from our own colony, and were housed at a constant temperature of 76°F. The mice were observed for 72 hours following injection. It would appear that the para-hydroxyl group on the cyclohexyl ring reduces toxicity very significantly, particularly as indicated by the marked

difference in toxicity between O-4,1172 and O-4,350, where the only structural difference is the presence of an hydroxyl on the ring of the latter compound. The toxicity of compounds O-4,1171 and O-4,1172 has been described previously by Lands, *et al.* (2).

The determination of stimulation of the central nervous system by these compounds was made by Frick and Becker of the Sterling-Winthrop Research Institute, according to the method of Schulte, Tainter and Dille (4). The data in table 2 was furnished through their courtesy. Amphetamine has been included as a reference compound. The results obtained indicate that, in doses of 20

TABLE 3

*Central nervous system effects of several orally active vasopressor compounds
(Method of Schulte, Tainter and Dille)*

COMPOUND	DOSE*	NUMBER OF EXPERIMENTS	RESULTS NUMBER OF REVOLUTIONS PER HOUR					
			1	2	3	4	5	6
Normal	<i>mgm /kgm</i> None	62	6.5	3.2	3.1	3.3	3.3	3.0
O-4,2†	5.0-80.0		No appreciable increase over normal					
O-4,1171	2.5	6	10.0	6.0	2.0	2.0	—	—
	5.0	12	39.0	28.0	9.0	5.0	5.0	2.0
	10.0	6	33.0	40.0	17.0	9.0	3.0	3.0
O-4,1172	20.0	6	15.0	6.0	2.0	5.0	3.0	1.0
O-4,345	20.0	6	7.1	2.3	2.5	3.5	2.0	2.0
O-4,350	20.0	6	9.1	0.8	1.5	4.5	3.5	—
Amphetamine sulfate	1.0	12	14.1	6.5	3.3	3.5	—	—
	2.5	12	30.0	31.0	12.4	4.0	—	—

* The salts in aqueous solution were injected subcutaneously.

† Schulte, *et al.* (8).

mgm./kgm., all the compounds, except O-4,1171, have but slight stimulating properties. Compound O-4,2 has no significant stimulating effect on the central nervous system at any dosage short of toxic levels. In the case of compounds O-4,1171 (l-isomer) and O-4,1172 (d-isomer) saturation of the ring has abolished or greatly diminished the central nervous system stimulation as compared with that of its phenyl homologue, desoxyephedrine, as reported by Schulte, *et al.* (8), and Lands, *et al.* (2). Compounds O-4,345 and O-4,350, the cyclohexyl homologues of 'Paredrine' and 'Paredrinol,' respectively, are somewhat weaker vasopressors than the corresponding phenyl analogues in anesthetized animals, but O-4,350 is a comparatively strong pressor drug when given orally. Neither

of these cyclohexyls has any significant central nervous system stimulating effects.

Figures 1 and 2 present a graphic description of average pressor activity, with simultaneous recordings of heart rate. In figure 1, the two isomeric compounds O-1,1171 and O-1,1172 are compared with O-1,2. With three times the dose, the isomers demonstrated a much shorter induction phase than did O-1,2, reaching their maximal activity in a significantly shorter time. This is also the case in figure 2, where O-1,345 and O-1,350 show a similar characteristic. However, it will be noted that when the effects of the cyclohexyl derivatives have declined to blood pressure levels which are of little significance, the effects of compound O-1,2 are still definite. In a few experiments where determinations were taken for longer periods of time than are indicated on the charts, O-1,2 showed blood pressure effects at levels significantly greater than normal for over four and one-half hours. Since all the compounds studied were long acting vasopressors, a study of the tachyphylactic effects was not carried out, although in some cases the compounds were administered on several consecutive days without alteration of the response.

Heart rates decreased in all cases, simultaneously with the rise in blood pressure. Since the blood pressure changes are all of approximately the same magnitude, it would seem that the heart rate changes are a direct reflex effect of the pressure rise. It should be noted that at no time were the heart rates lowered to levels suggesting toxic effects.

Discussion. The investigation of orally active vasopressor compounds, notably amphetamine, has been extensively described by numerous workers, including Chen, *et al.* (3), Piness, *et al.* (5), Schaumann (6), Hartung and Munch (7), Tainter (1) and many others. Prolonged pressor activity following oral administration has been indicated as a desirable property for such a compound. Several orally active substances are commercially available. However, certain undesirable side effects, principally due to central nervous system stimulation, have been described for them.

In the compounds tested, both oral activity and prolongation of vasopressor effects appear to involve the presence of the methyl group on the carbon of the side chain nearest to the N, as it is constant in all members of this series. Central nervous system stimulation seems to bear some relation to optical rotation, especially in the case of amphetamine, 1-amphetamine being about one-eighth as stimulating as d-amphetamine, according to Schulte, Reif, Bacher, Laurence, and Tainter (8). Further examination of the data of these workers indicates that many phenyl derivatives that possess vasopressor effects are also significantly stimulant upon the central nervous system. The most active are amphetamine and methyl-amphetamine, but in sufficiently large doses 'Propadrine,' ephedrine, 'Paredrinol' and 'Paredrine' also showed central nervous system stimulation. Frank, Altschule and Zamcheck (9) report that 'Paredrine' administered to dogs in post-hemorrhagic shock produced an increase in alertness and activity. From the data given in table 2, the compounds tested that possess a

saturated ring exhibit little or no central nervous system effects. This is particularly evident when there is hydroxyl substitution on the ring, as in compounds O-4,350 and O-4,345. Compounds O-4,1171 and O-4,1172, however, seem to retain some central nervous system effect, although this is greatly diminished. Saturation of the ring in this group of compounds produced a decrease in vasopressor potency. All the cyclohexyl compounds reported here, however, retain a significant proportion of their vasopressor efficiency. Lands, *et al.* (2), show that the racemic form of compounds O-4,1171 or of O-4,1172 is about two-thirds as active on blood pressure as the racemic mixture of the corresponding phenyl analogue. In the case of compounds O-4,345 and O-4,350 saturation of the ring produces a more marked loss of pressor effects. Compound O-4,345 has about one-sixth the activity of its phenyl analogue, while O-4,350 possesses about a quarter of the effect of its phenyl analogue. Neither of these compounds (O-4,345 and O-4,350) possesses any central nervous system activity in doses producing marked vasopressor effects.

In evaluating the data presented, emphasis should be placed upon the results obtained with unanesthetized dogs, as this is perhaps the most reliable criterion of oral effectiveness. Both the slower onset of maximum effects, and the more prolonged activity of compound O-4,2 over the others indicate that it is perhaps more slowly absorbed from the gastrointestinal tract, and perhaps less quickly destroyed in the body.

While it seems that the phenyl compounds are experimentally more effective pressor agents than the cyclohexyl compounds, saturation of the ring has produced compounds which have not only significant prolonged vasopressor activity when administered orally, but possess minimal central nervous system stimulation.

SUMMARY

1. Saturation of the ring of several phenyl compounds to produce cyclohexyl isopropylamines results in compounds which possess prolonged vasopressor effects when given orally.

2. Saturation of the ring results in some diminution of vasopressor effects; the activity of the compounds ranges from one-sixth to two-thirds that of their phenyl analogues when given intravenously. However, these differences were considerably less evident when the drugs were administered orally.

3. The cyclohexyl compounds described here exhibited the unexpected characteristic of being generally much less stimulating upon the central nervous system than their phenyl analogues.

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ACUTE AND CHRONIC TOXICITY OF AN ALIPHATIC AMINE, METHYLAMINO-ISO-OCTENE (Octin)¹

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Palmer (1) and, more recently, MacNeal and Davis (2) have reported symptomatic relief in some cases of migraine headache following the use of octin (methylamino-iso-octene or 6-methylamino-2-methyl-2-heptene). This synthetic, aliphatic amine has been used for some time in Europe and in this country as an antispasmodic chiefly for the relief of ureteral spasm (3, 4).

The drug is basically representative of several other simple aliphatic amines now being used also as topical vasoconstrictors and as cardiovascular stimulants. Their literature has been cited in the recent publications by Swanson and Chen (5) and by Jackson (6).

This more recent use of octin in the management of migraine has emphasized the necessity for a systematic study of possible toxic effects under more chronic conditions. At the time these experiments were begun there was no extensive study of this sort for any of this group of drugs. More recently, the distribution and excretion characteristics of octin have been determined (7) and chronic as well as acute toxicity studies have been carried out with two related drugs, 2-methyl-aminoheptane (8), 2-amino-6-methyl heptane (8a) and 2-methylamino-6-hydroxy-6-methyl heptane (Aranthol) (9).

ACUTE EXPERIMENTS. A series of acute experiments are presented in table I. These observations were made at approximate room temperatures of 24° to 26°C. All parenteral doses were administered in the form of the hydrochloride salt and all oral doses as the mucate salt. The content of base in these two salts is 79.5% and 57.4% respectively. The animals used were Swiss mice, albino rabbits and mongrel dogs all of medium weight. Estimated figures for LD₅₀ were calculated according to the procedure described by deBeer (10). The figures for Standard Error of LD₅₀ are taken as being equal to one-fourth of the difference between the upper and lower limits thus obtained.

With dogs, doses of 40 mg. per kg. intravenously usually caused spasm, particularly of the neck and jaw muscles, prompt disappearance of pulse and, in some cases, urination and defecation; artificial respiration ordinarily did not restore or maintain the heartbeat for any significant period. With doses of 30 mg. per kg. results were essentially the same but not as prompt. In the one animal which survived, there were tonic and clonic spasms, tremors and hyperpnœa for about 40 minutes. With doses of 20 mg. per kg. intravenously, the one death in 11

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TABLE I

DOSE	MORTALITY RATIO: NO. KILLED/ NO. IN GROUP	MORTALITY PERCENTAGE	DESCRIPTION
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<i>Intravenous administration of the hydrochloride</i>			
Mice			
mg./kg.			
60	5/5	100	Deaths in 15 to 30 sec. without much spasm
30	5/5	100	Convulsions in all cases; deaths in about 30 sec.
20	3/5	60	Deaths in 30 to 90 sec.
15	4/10	40	Deaths in 30 to 90 sec.; in survivors, tremors, spasms, hyperpnea
10	0/5	0	Moderate to marked effects
7.5	0/10	0	Moderate effects
Estimated LD ₅₀ in mg. per kg. = 17.5 (S.E. 2.7)			

Rabbits			
40	5/5	100	Deaths in about 1 min.
20	6/7	86	Deaths in about 2 min.
15	1/10	10	Considerable excitation; one death in 1½ min.
10	0/5	0	Moderate excitation
Estimated LD ₅₀ in mg. per kg. = 17.6 (S.E. 1.0)			

Dogs			
40	4/4	100	Deaths in 2 to 3 min.
30	4/5	80	Deaths in 2 to 4 min.
20	1/11	9	Marked cocaine-like excitation passing off in 60 to 80 min.
10	0/3	0	Moderate excitation
Estimated LD ₅₀ in mg. per kg. = 25.8 (S.E. 2.0)			

<i>Subcutaneous administration of the hydrochloride</i>			
Mice			
250	5/5	100	Deaths in 3 to 35 min. preceded by convulsions lasting about 3 min.
200	8/10	80	Deaths in 5 to 90 min.
150	2/10	20	Deaths in 5 to 10 min.
120	1/15	7	One death in 35 min.
80	2/10	20	Deaths in 5 to 20 min. Restlessness, occasional convulsions, rapid circling movements.
Estimated LD ₅₀ in mg. per kg. = 171 (S.E. 9.5)			

Rabbits			
120	4/5	80	Deaths in 15 to 60 min.
80	1/8	12.5	One death in about 5 hours; convulsions in 1 of the survivors
Estimated LD ₅₀ in mg. per kg. = 101 (S.E. 8.9)			

TABLE I—Continued

DOSE	MORTALITY RATIO: NO. KILLED/ NO. IN GROUP	MORTALITY PERCENTAGE	DESCRIPTION
Dogs			
<i>mg./kg.</i>			
100	3/4	75	Deaths in 90 to 240 min.
80	2/3	66	Deaths in 75 and 120 min.
60	0/4	0	Severe excitation in one case; prolonged excitation in others lasting about 8 hours.
40	0/3	0	Moderate to marked excitation; in some cases lasting 6 hours
20	0/5	0	Moderate to marked excitation; barely recognizable effects after 180 min.
10	0/4	0	Mild excitation, quiet in 40 min.
Estimated LD ₅₀ in mg. per kg. = 76.3 (S.E. 9.1)			
Oral administration (<i>mucate</i>)			
Dogs			
500	5/6	84	One survivor excited for more than 8 hours
200	3/5	60	Deaths in 40 to 200 min.
150	2/4	50	
100	3/8	37.5	Two deaths in 1 to 2 hours; 1 death over-night
75	0/8	0	Typical excitation lasting 2 to 8 hours; brief spasms in 2 cases
Estimated LD ₅₀ in mg per kg. = 148 (S.E. 55)			

trials occurred in one or two minutes without much spasm. During the excitation stage, head-jerking movements were characteristic. The excitation to a considerable degree resembled cocaine excitation in that there was mydriasis, hyperpnea, restless searching movements, licking, head shaking, ear twitching, tail wagging and exaggerated playfulness. Essentially the same type of excitation, but to a more moderate degree, occurred with doses of 20 and 10 mg. per kg. subcutaneously, the onset of symptoms usually becoming manifest in 3 to 30 minutes.

In rabbits receiving the larger intravenous doses, the effects were promptly manifested. The animals fell to the side and after a short interval, characterized by opisthotonus and severe spasm of the leg and trunk muscles, became limp and motionless. Respiration and heartbeat ceased at about the same time and artificial respiration did not significantly prolong the survival period. With the lower doses, the rabbits showed varying degrees of excitation with a lesser variety of manifestations and a shorter period of effects than the dogs.

In mice receiving the larger intravenous doses, the effects were even more prompt than in the case of the rabbits and dogs. With non-fatal intravenous doses, there were instances of tremors, spasms and hyperpnea for a relatively brief period. With the larger subcutaneous doses in mice, effects were deferred

in some cases for periods up to 90 minutes; fatal doses were preceded by periods of convulsions usually lasting not more than 3 minutes. In the case of some of the non-fatal subcutaneous doses there would be intervals characterized by very rapid circling of the cage.

In the case of oral doses, which were given only to dogs, the drug was administered in 10% water solution by stomach tube. Although vomiting occurred in some cases, it did not usually occur until 20 to 30 minutes after administration (except in one case which was rejected from the series) and did not seem to influence the results in that the dogs which vomited usually exhibited more severe effects than the other dogs in the same dosage range. Vomiting appeared largely to be a central effect taking place after the major portion of the drug had been absorbed. Emesis also occurred in some of the dogs receiving the drug parenterally.

Some general interpretations can be drawn from this series of single doses in intact animals. Mice and rabbits are more sensitive than dogs to intravenous injections. Mice, on the other hand, are much less sensitive than dogs to subcutaneous injections; rabbits are intermediate in this respect. A possible explanation may be based on the presumption that deaths from intravenous injections are due predominantly to cardiac depression and deaths from subcutaneous injections are more dependent on central nervous system effects. Accordingly, it might be expected that the species with the more highly developed nervous system would show greater susceptibility under conditions in which nervous system effects are more prominent.

CARDIAC EFFECTS. The general appearance of the deaths caused by large, intravenous doses in mice, rabbits and dogs suggested that myocardial depression was a predominant factor. This was largely based on the prompt disappearance of the pulse, the continuation of respiratory movements for a period after disappearance of the pulse and the inefficacy of artificial respiration as a resuscitative measure. Accordingly, the cardiovascular effects of this drug were subjected to further analysis.

Perfusion of the isolated rabbit heart by a modification of the Langendorff-Martin perfusion arrangement was carried out in 14 experiments. In about half of these experiments, a series of perfusion concentrations of 1-500,000 to 1-50,000 in Ringer-Locke solution were used. At the more dilute range of this series, a very occasional stimulant effect was obtained on initial application; this could not be readily duplicated under the same conditions in other experiments. Ordinarily there was no stimulation or more commonly a definite depression. In other experiments intended to have more exploratory value, injections at varying rates were made directly into the perfusion fluid. The amounts ranged from 0.05 cc. to 0.5 cc. of a 1-1,000 solution. Stimulation was occasionally obtained with volumes of 0.1 cc. on initial injection, but the effect was not readily reproducible and the usual responses varied from no effect to sharp depression. With direct injection of variable quantities or with alteration of varying concentrations of perfusion fluid, depressant effects were consistently obtained with higher concentrations.

TABLE II

Circulatory effects in open-chest dog preparations (Pentobarbital anesthesia)

DOSE	NO. OF EXPERIMENTS	EFFECTS
Intravenous administration		
mg./kg.		
40	3	Immediate cardiac dilation and rapid fall in b.p. to zero level.
20	2	Similar but slower effects terminating in 15 min. No phase with increase in isometric systolic tension (I.S.T.).
10, 10, 10, 20, in 35 min. and in 53 min. (fig. 1)	2	After biphasic (depressor-pressor) b.p. response with 1st dose, only depressor responses with subsequent doses. I.S.T. decreased with each injection as b.p. fell and increased only during pressor phase of 1st dose. Heart dilated progressively and finally stopped.
5 given 8 times in 140 min.	1	Biphasic b.p. response and increase in I.S.T. and heart rate with 1st dose. Subsequent doses produced only depressor effects with decreases in heart rate and I.S.T. Heart activity and b.p. progressively fell to zero.
2.0 given 5 times in 60 min.	1	Biphasic b.p. response and increase in I.S.T. with first 3 doses. Purely depressant effects in subsequent doses. Heart activity and b.p. at good level when experiment terminated.
1.0 given 12 times in 145 min. (fig. 2)	1	Biphasic b.p. responses with first 5 doses, in which pressor phases progressively decreased. Only depressor phases with subsequent doses. Distinct increases in I.S.T. with first doses progressively diminishing until absent after 6th dose. Substantial decreases in I.S.T. with depressor phase of all injections subsequent to 6th injection. Heart active and b.p. at moderate level when experiment terminated.
1.0 given 25 times in 220 min.	1	Biphasic b.p. responses with first 15 doses, in which pressor phases progressively decreased. Only depressor phases with subsequent doses. Distinct increases of I.S.T. with pressor phases of first 2 doses; no increases in I.S.T. with succeeding doses. Heart active and b.p. at moderate level when experiment terminated.
0.5 given 12 times in 120 min.	1	Essentially the same type of effects as in above experiment with 1.0 mg./kg. doses. No pressor phase or increase in I.S.T. after 8th dose.
0.2 followed by 20 given 4 times in 60 min. (fig. 4)	1	Pressor response and increase in I.S.T. with 0.2 dose, progressive depressor effects with doses of 20; cardiac standstill with last dose.
Intramuscular administration		
15.0 given 5 times in about 120 min. (fig. 3)	4	With the first dose, very slight or no depressor phase, considerable increase in I.S.T., b.p. and heart rate, reaching peak in about 5 min. and usually returning to normal in about 30 min.; with succeeding doses either no effect or moderate depression of I.S.T., b.p. and heart rate. Heart active and b.p. at functional level when experiments +

Ahlquist (11, 12) has previously described effects on blood pressure, pulse pressure and respiration of octin in doses of 0.1 to 10 mg. per kg. intravenously in dogs. According to his reports, doses of 1.0 mg. per kg. or more produced a preliminary hypotensive or depressor phase followed by a hypertensive or pressor phase; with successive injections, the hypotensive phase progressively predominated. With doses of 0.1 to 0.2 mg. per kg. depressor responses did not usually occur. On the basis of pulse pressure observations, he considered that the hypotensive phase with larger doses was probably due largely to direct myocardial depression and Jackson has expressed a similar view in the case of a closely related amine (13). Subsequent observations by Ahlquist, using a special flow-meter, have shown that there is also a vasodilating component in this hypotensive phase (14).

Our observations obtained in open-chest dog preparations are summarized in table II. The results are compatible with the above mentioned observations of Ahlquist and of Jackson and, additionally, define the variable degree of myocardial depression or stimulation as obtained under some of these conditions. The contractile force of the heart was determined according to a method previously described, by means of which the isometric systolic tension of a section of the right ventricle is determined and the result expressed in grams (15).

Results summarized in table II as well previous observations demonstrate that, according to the dose, there may be either vasodilation or vasoconstriction, heart slowing or acceleration and myocardial depression or stimulation. The balance of these factors shifts as small or moderate doses are successively repeated, the direction of change resulting in lowered blood pressure, contractile force and rate. With single intravenous doses of 1.0 mg. per kg. and above, blood pressure responses are biphasic in that the blood pressure both falls and rises above that of the preceding control period and there may also be variations in contractile force of the heart with both distinct depression and distinct stimulation as components of the response to a single injection.

With regard to dose ranges which are fatal to the heart under these conditions of mechanical insufflation with oxygen, single, intravenous doses of 20 mg. per kg. and above may promptly prove fatal. (In one of the recorded experiments, however, four such doses were required to bring the heart to standstill.) If intravenous doses of 40 to 50 mg. per kg. are given in instalments over periods of one-half to 2 hours, they also may be progressively fatal. Intravenous doses of 1.0 mg. per kg. may be repeated up to 25 times over periods of 3 to 4 hours without terminating the experiment. When the intramuscular route is used, much larger doses may be given without fatal cardiac depression; by this route, doses of 15 mg. per kg. produce on first administration pure stimulation without depressor effects; 5 such successive injections, totalling doses of 75 mg. per kg. do not ordinarily stop the heart. The recommended clinical dose is 1.5 to 3.0 mg. per kg. intramuscularly or subcutaneously and, accordingly, it might be expected that the usual response to such injections would be that of minimal to moderate hypertensive effects. This corresponds to the experiences reported by MacNeal and Davis (2).

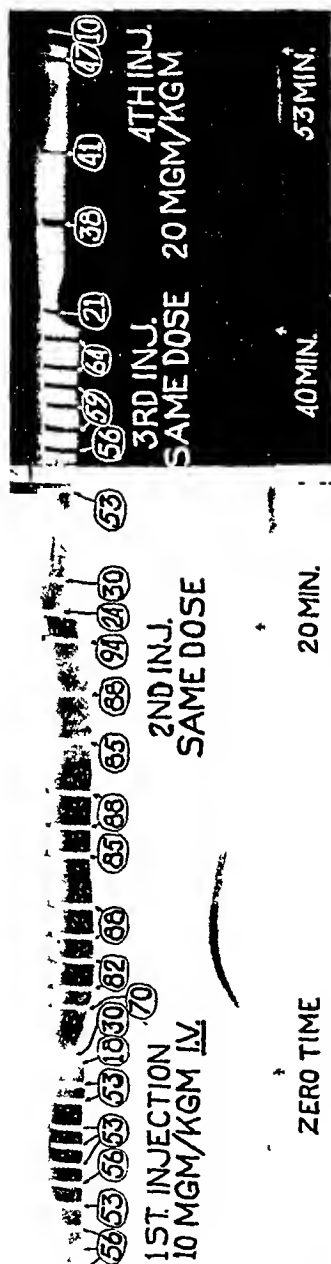


FIG 1 UPPER TRACING—RECORDING HEART LIVER WITH DOWN STROKE REPRESENTING SYSTOLE
Encircled figures represent estimated contractile force expressed in grams or isometric systolic tension (I. S. T.).
Lower tracing - arterial pressure with Hg manometer

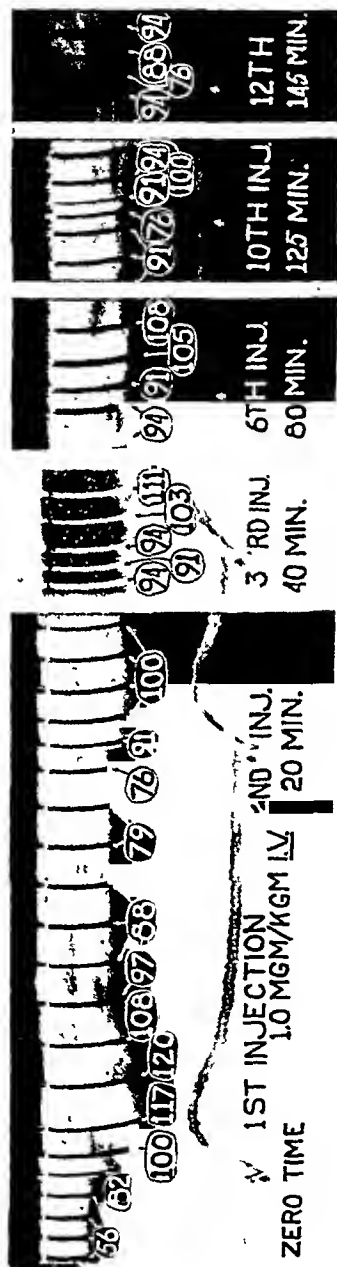


FIG 2 SAME RECORDING ARRANGEMENTS AS IN FIG. 1

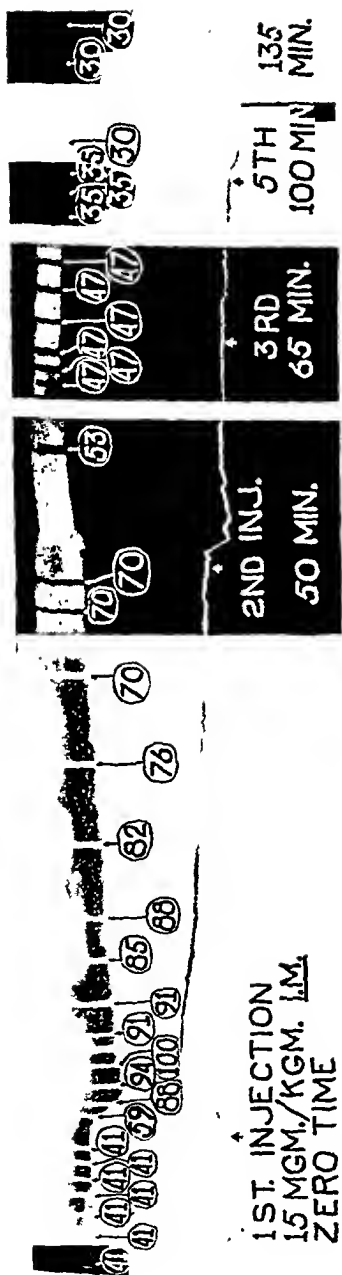


FIG. 3. Saw Recording Arrangements as in Figs 1 and 2

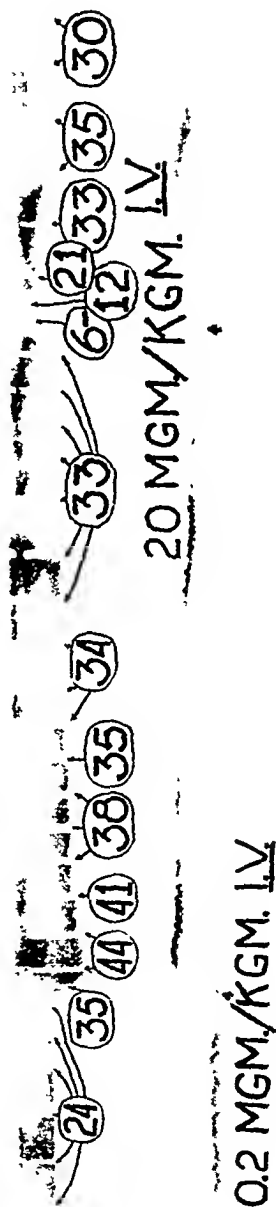


FIG. 4. SAME RECORDING ARRANGEMENTS AS BEFORE

These 2 doses approximately represent the extremes giving solely pressor and stimulant effects in the instance of the lower dose and solely depressant effects in the instance of the larger dose. The intravenous dose of 0.2 mg./kg. corresponds approximately in type of effect with that obtained in fig. 3 with an initial dose of 15 mg./kg. given intramuscularly.

CHRONIC EFFECTS. Octin was administered over extended periods of time to mice by drinking water, to dogs by capsule and by subcutaneous injection, and to roosters by intramuscular injection. Doses in some cases were near the levels producing manifestations of intense cerebral excitation and this appeared to be the only limitation of chronic administration. If the doses reached this level there was hyperactivity, lowered food intake and corresponding weight loss. If the dosage was kept below this level, no consistent or characteristic drug effects in any of the groups were observed with respect to general appearance, growth curves, reproduction, blood picture or gross and microscopic autopsy.

With mice, the medium of drinking water was preferred because of the greater reliability of dosage figures. (The advantages of this method over the use of food-drug mixtures has been recently discussed by Kramer and Bunch (16).) Mice, however, refused concentrations of 0.1% of the mucate even through stages of dehydration; a concentration of 0.025% was used and this was accepted about as readily as plain water. Although this concentration desirably might have been higher, it represented an average daily intake of approximately 40 mg. per kg., and thus was about 6 times the daily oral dose (per kgm.) which has been used in the treatment of migraine (1).

Two groups of Swiss mice numbering 15 each were maintained for 180 days on tap water in one case and on 0.025% octin mucate aqueous solution in the other. During the course of this time they matured, were bred, and after maturity of the litters, they in turn were bred. In another trial, mice of the C₃H strain (all males) were divided into 2 groups of 10 and 20 each. They were maintained for 195 days on tap water and 0.025% octin mucate solution, respectively. In the case of both trials, the average daily intake was approximately 40 mg. per kg. and, accordingly, the final total ingested drug was 7.2 gms. per kgm. in the case of the Swiss mice, and 7.8 gm. per kg. in the case of the C₃H mice.

The use of medicated drinking water with dogs was not satisfactory. With an increase in room temperature, the water intake, in some instances, increased enough to raise the dosage to the point of cerebral excitation and this in turn provoked further drinking of water, which resulted in acute, severe excitation. The procedure finally adopted consisted in the twice daily administration of capsules containing 30 mg. per kg. This at times produced some slight degree of excitation which was not disturbing. About the same degree of excitation occurred in doses of 4 mg. per kg. given subcutaneously.

The schedule of administration to dogs is shown in table III.

Three roosters were injected intramuscularly with single daily doses of 20 mg. per kg. of octin hydrochloride over periods of 66 to 132 days. This dose produced a clearly recognizable degree of acute excitation which, however, did not appear to interfere with growth. From the same brood, one rooster was maintained as a control and two were injected daily with fluid extract of ergot and later with ergotamine tartrate (1-1,000) in daily doses of 1 cc. per kg. The fluid extract of ergot appeared to be lacking in chronic effects over the period of its use (44 days); when this was followed by the ergotamine, the comb tips became gangrenous in 5 days. Acute effects on the combs usually consisted of blanching

at the base with cyanosis at the tips. Such effects at these doses were least marked with octin, more pronounced with fluid extract of ergot and very marked with ergotamine tartrate.

Gross autopsies and examination of approximately 300 tissue sections following this chronic administration in mice, dogs and roosters failed to show any significant type of tissue damage which could be attributed to the drug administration. Special attention was given to tips of the mice tails and to the combs of the roosters, which, however, showed no indication of gangrene.

In the dogs some special attention was given to occasional abnormalities in the kidneys, which suggested a limited degree of glomerulo-nephritis in some cases and interstitial nephritis in others. The lesions were of a non-specific type and bore no relation to the dosage of the drug. Urine examinations toward the end of the medication period were consistently negative for albumin. The

TABLE III
Schedule of chronic administration in dogs

DOG NO.	STARTING WEIGHT	FINAL WEIGHT	DAILY DOSE	NUMBER OF DAYS	TOTAL DOSE
Oral administration (Δ lucate)					
	kg.	kg.	mg. per kg.		gm. per kg.
1	2.4	5.5	60	112	6.7
2	5.4	9.3	60	170	10.2
3	0.9	7.2	5-60	316	13.2
Intramuscular administration (Hydrochloride)					
4	7.0	13.0	4	167	0.7
5	4.1	8.8	4	212	0.8
6	4.6	8.2	4	217	0.9

conclusion was reached that these lesions were of the same type as has been observed in ordinary kennel dogs without medication. Morehead and Little (17) described similar findings in 11 of 20 such dogs. Bloom (18) found 108 dogs with lesions of focal interstitial nephritis among 200 dying of various causes without uremic symptoms. MacNider (19) reported nephropathy in 42 dogs from a group of 237. Before concluding that the lesions observed in our examinations were unrelated to drug effects, consideration was given to the fact that vinyl and iso-allyl amines have both been shown to cause a specific necrosis of tubular epithelium (20-25). Allyl amine, however, has been reported in some of these studies as being without such effects (25).

Relevant chronic studies which may be cited are those by Shaffer (8) who administered 2-methylaminoheptane to rats and obtained growth interference with doses of 23 mg. per kg. daily in females and 122 mg. per kg. daily in males. This latter dose did not interfere with fertility or give evidence of histologic damage with either the males or the females. Chen (26) reported the chronic administration of ephedrine by various routes in rats and rabbits giving special

attention to the possible development of arteriosclerosis. No significant growth retardation or tissue damage was noted. Doty (27) also carried out such experiments with larger doses of ephedrine and observed no significant effects on tissue sections. MacNeal and Davis (2) have described the instance of one patient who received 113 injections of octin in doses of 150 to 200 mg. over a period of 15 months.

SUMMARY

(Dosages in the following represent the dose per kg. of body weight.)

1. Methylamino-iso-octene hydrochloride administered intravenously was found to have an LD_{50} of 17.5 mg. (S.E. 2.7) in mice, of 17.6 mg. (S.E. 1.0) in rabbits and of 25.8 mg. (S.E. 2.0) in dogs. Administered subcutaneously, the estimated LD_{50} was 171 mg. (S.E. 9.5) in mice, 101 mg. (S.E. 8.9) in rabbits and 76.3 (S.E. 9.1) in dogs. Administered orally to dogs the mucate salt was found to have an estimated LD_{50} of 148 mg. (S.E. 55).

2. The acute manifestations in dogs, resembled, in several particulars, those of cocaine excitation; characteristic manifestations in rabbits and mice were less conspicuous.

3. On the isolated rabbit heart, the most consistent effect was a temporary depression. Circulatory effects in the open chest dog preparation are primarily depressant in the dose range of 20 to 40 mg. intravenously. In the dose range of 0.5 to 10 mg. intravenously effects are biphasic (depressor-pressor) with respect to blood pressure; they are similarly biphasic with respect to contractile force of the heart after repetition of the smaller doses in this range or with the first of the higher doses in this range. Initial intramuscular doses of 15 mg. per kg. produce only stimulation of rate and contractile force with an increase in blood pressure.

4. Prolonged administration of octin hydrochloride to roosters in daily doses of 20 mg. intramuscularly failed to produce gangrene of the comb, while ergotamine tartrate readily produced gangrene of the comb tips with other roosters from the same brood.

5. On chronic administration, no significant effects were observed in mice receiving octin mucate in average daily doses of 40 mg. orally for periods up to 195 days (total 7.6 gm.), in dogs receiving the mucate at an approximately similar rate for periods up to 316 days (total 13.2 gm.), and in dogs receiving daily doses of 4 mg. of the hydrochloride intramuscularly for periods up to 127 days (total 900 mg.).

CONCLUSION

In so far as such animal experiments are indicative, the acute effects of methylamino-iso-octene represent the most prominent limitation to its administration in that significant or cumulative effects were not obtained within the dosage range of this study.

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STREPTOMYCIN IN THE BLOOD: CHEMICAL DETERMINATIONS AFTER SINGLE AND REPEATED INTRAMUSCULAR INJECTIONS

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In the study of chemotherapeutic agents, measurement of the concentrations attained in the blood after systemic administration provides information of value in determining time-dose relationships. Numerous investigations of this type have been carried out with streptomycin employing microbiological assays, and clinically effective dosage regimens have been formulated. It has been generally recognized, however, that the numerous inherent and technical variables of the biological methods impose serious restrictions on attempts at precise analysis of the results obtained.

Recently a chemical method for the determination of streptomycin in blood and cerebrospinal fluid has been reported (1). The method is technically simple, with a standard deviation of 6 per cent for a single determination, and has a lower limit of sensitivity of 1.0 microgram in 1 ml. of serum or plasma. A comparison was reported [see Table II of (1)] of the results obtained by this chemical method with a microbiological technique employing a paper disc modification of the procedure of Stebbins and Robinson (2). In a series of 45 samples of human plasma, containing from 2.5 to 33 γ of streptomycin per ml., the microbiological assay values were found to be $105 \pm 14\%$, if the chemical values were arbitrarily taken as 100%. The numerous advantages of the chemical method of measurement of streptomycin made it appear desirable to reinvestigate the concentrations of streptomycin in the blood of dogs and humans following intramuscular administration.

METHODS AND MATERIALS. Two highly purified samples of streptomycin were used in these experiments; a streptomycin trihydrochloride with a potency of 700 γ /mg. and a crystalline streptomycin trihydrochloride calcium chloride double salt, 730 γ /mg. The crystalline salt was used for all the human subjects, while both preparations were administered to the dogs. Intramuscular injection was employed in all instances. Aliquots of the injection solutions were assayed by two chemical methods, the maltol reaction method (3) and the hydrazine method (1). The doses reported in the tables were based on these assays.

As it was shown in previous papers (1, 4) that no significant amounts of streptomycin permeate the erythrocyte, the drug concentration was determined in plasma or serum.

Blood samples were obtained routinely just before injection of the drug, in order to obtain accurate plasma blanks for the chemical measurement. The small variations in the plasma blanks were of analytical significance only if concentrations of streptomycin of less than 5 γ /ml. were to be measured with high accuracy. All samples were stored at refrigerator temperature (6°C) and analyses were performed within one week. In control experiments it was established that no deterioration of streptomycin occurred during this time.

RESULTS. 1. *Streptomycin concentrations in the blood following a single injection.* a) *DOG.* In order to measure the rate of decrease of streptomycin

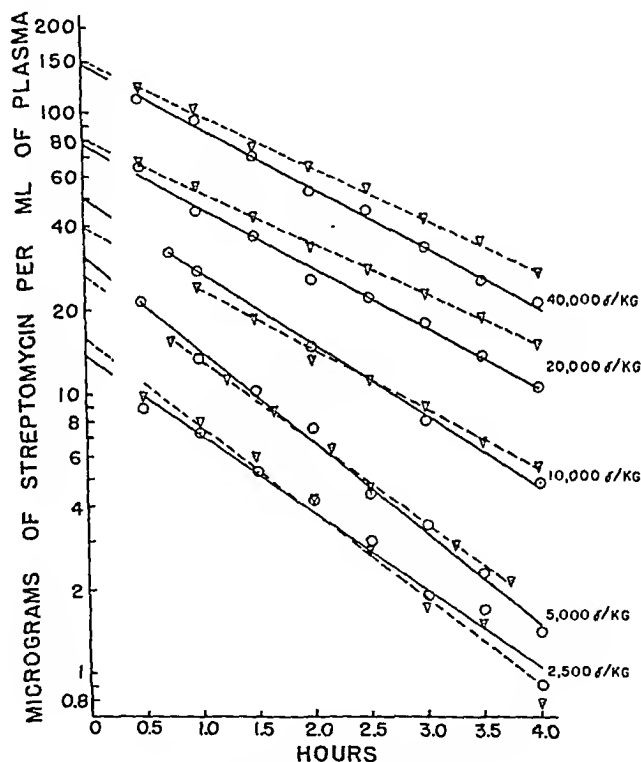


FIG. 1. STREPTOMYCIN CONCENTRATIONS IN THE PLASMA OF DOGS AFTER SINGLE INTRAMUSCULAR INJECTION

in the plasma of dogs, two animals were each injected intramuscularly with a single dose, except in one case when four dogs were used. The doses were based on body weight and ranged from 2,500 to 40,000 γ /kg. Blood samples were obtained at half hour intervals for a period of four hours.

The curves obtained by plotting the plasma concentration against time were exponential after the first half hour, since a straight line resulted on plotting the logarithm of the plasma concentration against time (fig. 1). The peak values

in the dog were usually reached within the first 30 minutes after injection. In two instances out of sixteen experiments, the peak was reached between 30 and 60 minutes.

To express the graphical presentation of figure 1 numerically for comparative interpretations, the slope K , and the intersect C_0 , of the straight lines extrapolated to zero time were determined by the method of least squares. These data, together with their standard deviations, are presented in Table I. In 90 percent of the cases the deviation was not more than 6 per cent from the straight line and in no case more than 10 per cent.

Extrapolation of the straight lines to zero time represents a concentration C_0 , which will be referred to as the "extrapolated maximum concentration."

TABLE I

Rate of decrease of streptomycin concentration in plasma of dogs after single intramuscular injection

$$\log C = K.t + \log C_0$$

DOG NO.	WT.	STREPTOMYCIN INJECTED	C_0 (EXTRAPOLATED)	VOL. OF DISTR. AS % OF BODY WT.	C_0 CALCD. FOR A DOSE OF 10,000 $\gamma/\text{kg.}$	K	$t_{1/2}$
	kg.	$\gamma/\text{kg.}$	$\gamma/\text{ml.}$	%	$\gamma/\text{ml.}$	hours ⁻¹	hours
813	12.4	40,000	148.3 \pm 6.2	27.0 \pm 1.1	37.0	-0.216 \pm 0.005	1.40 \pm 0.03
834	12.5	40,000	153.8 \pm 6.2	26.0 \pm 1.0	38.4	-0.188 \pm 0.005	1.60 \pm 0.04
799	11.8	20,000	77.5 \pm 4.6	26.0 \pm 2.0	38.7	-0.218 \pm 0.007	1.38 \pm 0.05
732	11.3	20,000	81.9 \pm 2.1	24.4 \pm 0.6	40.9	-0.186 \pm 0.004	1.62 \pm 0.03
766	12.8	20,500	87.1 \pm 0.2	23.4 \pm 0.1	42.5	-0.180 \pm 0.001	1.67 \pm 0.01
732	13.2	20,500	83.8 \pm 2.3	24.6 \pm 0.7	40.7	-0.180 \pm 0.008	1.68 \pm 0.07
732	13.8	10,000	39.3 \pm 1.3	25.5 \pm 0.9	39.3	-0.217 \pm 0.005	1.39 \pm 0.04
579	11.9	10,000	50.6 \pm 1.5	19.7 \pm 0.6	50.6	-0.259 \pm 0.005	1.16 \pm 0.02
580	11.7	5,000	31.0 \pm 2.4	16.2 \pm 1.2	62.0	-0.328 \pm 0.009	0.92 \pm 0.02
832	7.7	5,000	26.7 \pm 1.3	18.7 \pm 0.9	53.4	-0.297 \pm 0.007	1.01 \pm 0.02
580	11.9	2,500	13.8 \pm 1.5	18.2 \pm 2.0	55.2	-0.278 \pm 0.016	1.08 \pm 0.06
579	11.9	2,500	15.8 \pm 1.6	15.8 \pm 1.6	63.3	-0.309 \pm 0.013	0.97 \pm 0.04

The "apparent" volume of distribution was calculated by dividing the total dose injected by C_0 and is recorded in Table I as percentage of body weight. To test proportionality of dosage per kg. to blood concentration attained, the C_0 values were converted to the basis of the same dose, 10,000 $\gamma/\text{kg.}$ These values are recorded in the column " C_0 calculated for a dose of 10,000 $\gamma/\text{kg.}$ " From the slope of the straight line K , the time for the streptomycin concentration to decrease to one-half of any given value was computed.

The dose had a definite influence on both the rate constant for the decrease of streptomycin concentration and the "apparent" volume of distribution. At the low doses, 2,500 and 5,000 $\gamma/\text{kg.}$, a higher rate constant and smaller volume of distribution were found than at 20,000 and 40,000 $\gamma/\text{kg.}$ The "apparent" volume of distribution, about 25 per cent, found with the higher doses is only slightly larger than the value usually accepted as representing the total extra-

cellular fluid (ca. 20 per cent). For these higher doses the average value for the concentration C_0 , calculated for every 10,000 γ /kg. injected was 39.6 γ /ml.

b) *MAN*. Similar studies were performed on 18 hospitalized adults who showed no apparent excretory abnormality. The doses ranged from 1600 to 20,000 γ /kg. Blood samples were obtained at specific intervals, usually for four consecutive hours and in some cases over a period of 7 hours.

In figure 2 the results were plotted on hemilogarithmic coordinates in the same manner as for the dog. The experimental data of only nine of the eighteen patients are presented, since some of the lines were superimposed.

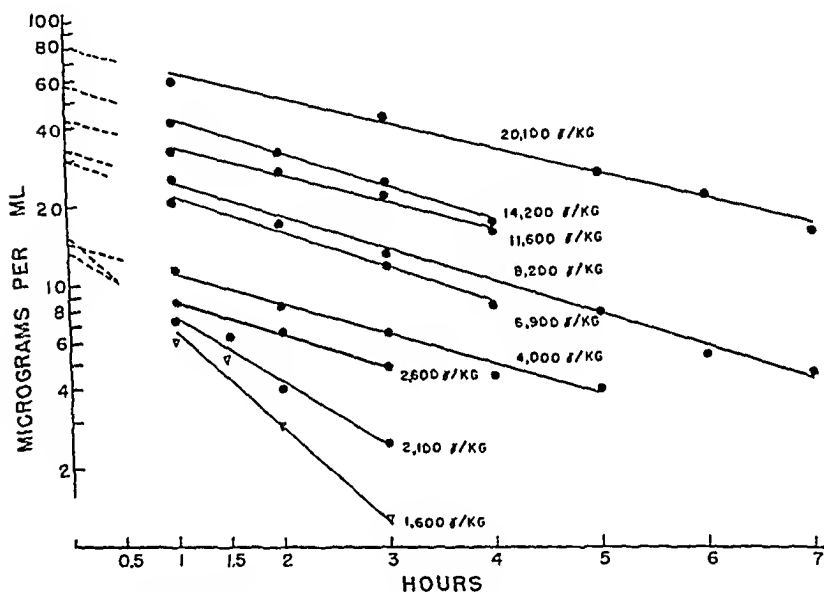


FIG. 2. STREPTOMYCIN CONCENTRATIONS IN HUMAN SERUM AFTER SINGLE INTRAMUSCULAR INJECTION

The results in man were qualitatively similar to those obtained in dogs, but there were some quantitative differences. From the slope of the lines it is apparent that the rate constant in man is lower than in the dog, and the actual peak was reached later than in dogs, appearing from 30 to 60 minutes after injection in 15 cases, while in 3 cases it occurred after 1 hour.

The values in Table II were calculated from the experimental data on man by the same methods used in Table I for the experiments with dogs.

The values fall into two distinct groups. In the patients who received 4,000 to 20,000 γ /kg., the response as indicated by the various data was quite regular. With doses of less than 4,000 γ /kg., however, irregular results were obtained.

Within the range of 4,000 to 20,000 γ /kg., the serum concentrations of strepto-

mycin were proportional to the dose administered. The proportionality within this dosage range may be seen in the data of Table II, particularly in the column " C_0 calculated for a dose of 10,000 $\gamma/\text{kg.}$ " and the related "apparent" volume of distribution. The slope K , and the half-life are also quite consistent within this range.

TABLE II

Rate of decrease of streptomycin concentration in human serum after single intramuscular injection

$$\log C = Kt + \log C_0$$

PATIENT	WT.	STREPTOMYCIN INJECTED	C_0 (EXTRA- POLATED)	VOL. OF DISTR. AS % OF BODY WT.	C_0 CALCD. FOR A DOSE OF 10,000 $\gamma/\text{kg.}$	K	t_1
	kg.	$\gamma/\text{kg.}$	$\gamma/\text{ml.}$	%	$\gamma/\text{ml.}$	hours^{-1}	hours
Cr....	72.0	20,200	64.7 \pm 1.7	31.2 \pm 0.8	32.1	-0.064 \pm 0.002	4.70 \pm 0.15
Ke....	63.4	20,100	79.1 \pm 4.9	25.4 \pm 1.6	39.4	-0.097 \pm 0.006	3.12 \pm 0.17
Pe	60.6	14,200	56.9 \pm 1.5	25.0 \pm 0.6	40.0	-0.125 \pm 0.005	2.39 \pm 0.09
Bo . .	60.6	14,200	51.8 \pm 1.7	27.4 \pm 0.9	36.5	-0.124 \pm 0.006	2.43 \pm 0.11
Ke... .	62.6	13,900	63.0 \pm 0.6	22.2 \pm 0.2	45.0	-0.124 \pm 0.015	2.43 \pm 0.03
McC.	67.6	13,400	50.1 \pm 1.0	26.2 \pm 0.5	37.4	-0.103 \pm 0.003	2.92 \pm 0.09
Ra....	37.2	11,600	42.2 \pm 1.9	27.4 \pm 1.2	36.5	-0.102 \pm 0.009	2.88 \pm 0.18
To.. .	69.0	8,200	32.5 \pm 1.8	25.2 \pm 1.4	39.6	-0.125 \pm 0.005	2.40 \pm 0.09
Ka.....	62.2	6,900	29.8 \pm 1.7	23.4 \pm 1.3	43.2	-0.135 \pm 0.011	2.24 \pm 0.16
Gi.....	64.2	4,200	14.9 \pm 0.1	28.2 \pm 0.2	35.5	-0.101 \pm 0.002	2.97 \pm 0.07
Ti.....	82.6	4,000	14.4 \pm 0.9	27.4 \pm 1.7	36.3	-0.116 \pm 0.008	2.59 \pm 0.16
Pe . . .	57.0	3,800	14.0 \pm 1.0	27.0 \pm 1.9	37.1	-0.114 \pm 0.013	2.65 \pm 0.25
Co	61.4	2,900	17.6 \pm 0.2	17.0 \pm 0.2	58.8	-0.149 \pm 0.003	2.03 \pm 0.03
To..	78.2	2,600	11.5 \pm 0.2	22.6 \pm 0.4	43.8	-0.126 \pm 0.004	2.39 \pm 0.08
Ti.... .	87.8	2,500	10.8 \pm 0.7	23.5 \pm 1.5	42.4	-0.071 \pm 0.002	4.24 \pm 0.90
Fr...	52.6	2,100	13.4 \pm 1.2	15.3 \pm 1.4	65.3	-0.246 \pm 0.026	1.23 \pm 0.12
Br	60.0	1,700	5.7 \pm 0.2	29.9 \pm 1.1	30.5	-0.069 \pm 0.011	4.39 \pm 0.60
To.	68.6	1,600	15.2 \pm 1.1	10.4 \pm 0.8	97.0	-0.361 \pm 0.021	0.83 \pm 0.04

TABLE III

Comparison of averaged data for man and dog

	DOSAGE RANGE	VOL OF DISTR. AS % OF BODY WEIGHT	C_0 CALCD FOR A DOSE OF 10,000 $\gamma/\text{kg.}$	K	t_1
	γ/kg	%	$\gamma/\text{ml.}$	hours^{-1}	hours
Man... .	4,000-20,000	26.3 \pm 2.3	38.2 \pm 3.5	-0.111 \pm 0.019	2.72 \pm 0.47
Dog.....	10,000-40,000	25.3 \pm 1.2	39.6 \pm 1.8	-0.198 \pm 0.018	1.52 \pm 0.14

The values for the "apparent" volume of distribution, C_0 for a dose of 10,000 $\gamma/\text{kg.}$, K , and t_1 , obtained on the group of 12 patients receiving more than 4,000 $\gamma/\text{kg.}$ were averaged and are compared in Table III with similar average values calculated for the 7 dogs injected with doses from 10,000 to 40,000 $\gamma/\text{kg.}$

The "apparent" volume of distribution and the related figure of C_0 for a dose of 10,000 $\gamma/\text{kg.}$ were essentially the same for man and dog. The rate constant

for the decrease of streptomycin concentration, however, is appreciably lower in man than in the dog, resulting in a longer half-life for the streptomycin concentration in the blood of man.

From the data in Table II, it is possible to calculate with reasonable accuracy the streptomycin concentration in the serum or plasma any time after the first hour following a single intramuscular injection by substituting in the equation $\log C = K.t + \log C_0$. It should be noted that the value for C_0 in Table III is for a dose of 10,000 $\gamma/\text{kg.}$, and when any other dose within the range from 4,000 to 20,000 $\gamma/\text{kg.}$ is administered, the value for C_0 must be proportionally decreased or increased.

A comparison of these results with work previously appearing in the literature is difficult because of the variety of bioassays used and because in some cases the doses cannot be recalculated to a unit weight basis from the reported data. The experimental work on animals by Stebbins, Gracssle and Robinson (5) and the data on drug concentrations in the blood of man by Buggs, Pilling, Bronstein and Hirschfeld (6) are of the same order of magnitude and follow a similar course as in our experiments.

2. *Streptomycin concentrations in the blood after repeated injections.* In the use of many chemotherapeutic agents it is customary to administer drugs at regular intervals in an effort to maintain a therapeutically effective concentration in the blood at all times. It is possible to predict the course of blood concentration during repeated dosing if the rate constant K , and the relationship between the extrapolated maximum concentration C_0 , and the injected dose per unit weight are known.

For example, one hour after an initial injection the concentration of the drug has reached a certain value, and thereafter it decreases exponentially until the next injection is given. The concentration resulting from the second injection will be superimposed upon any residual drug from the previous dose. Since the rate of decrease is proportional to the concentration, the drug concentration in the blood will now decrease more rapidly, and the value just before the third injection may be only slightly higher than the one before the second injection. After several equally spaced injections of the same dose, depending on the rate constant and on the interval between injections, a substantially steady state will be reached, and if the procedure is then continued, the drug concentration in the blood will neither fall below a predictable concentration nor build up beyond a certain value. These considerations apply to any drug where the rate of decrease in the blood is experimentally found to be proportional to its concentration.

The general expression for such a case can be formulated. Let C_0 be the extrapolated maximum concentration at time zero; let K be the rate constant, if logarithms to the base 10 are used; let C be the drug concentration at any given time t ; and let T be the interval between doses, then:

$$(1) \quad \log C = K.t + \log C_0, \text{ or}$$

$$(1a) \quad C = C_0 \cdot 10^{Kt}$$

$$\text{Let,} \quad r = 10^{KT}$$

The blood concentration will be $C_0 r$ just before the second dose, and, theoretic-

cally, just *after* the second dose it will be $C_o + C_o r$, and again just *after* the third dose it will be, $C_o + C_o r + C_o r^2$, or after n doses:

$$(2) \quad C_n = C_o (1 + r + r^2 + \dots + r^{n-1})$$

Since r is less than 1, C_n approaches an upper limit as n increases, and by letting n approach ∞ , the sum of the geometrical progression can be substituted in equation (2) to give this limit. Thus,

$$(3) \quad C_\infty = C_o \cdot \frac{1}{1-r}$$

The preceding considerations are still applicable even if C_o is not determined by extrapolation. A similar equation can be used to make reasonably accurate predictions of actual concentrations provided that the values selected for computation lie within the period in which the rate of decrease is proportional to the concentration. Thus, if the foregoing provision is met, the concentration expected to be found experimentally at any time during an injection interval, after a steady state has been established, can be computed from the following equation:

$$(4) \quad C_H = C_A \cdot \frac{1}{1-r}$$

where C_A is the concentration at $t = h$, experimentally observed in the first injection interval, and C_H is the actual concentration to be expected at the corresponding time in any interval after a steady state has been established.

For experimental verification, a dog was injected with a dose of 20,500 γ /kg. every 3 hours and blood samples were taken at hourly intervals for 12 hours. A second experiment was similarly performed except that the time interval was reduced to 2 hours. The experimental results were plotted on hemilogarithmic coordinates in figure 3.

From figure 3 it is immediately evident that the experimental facts confirm the predictions. Repeated dosing did not produce unlimited building up of the blood concentrations, in spite of the fact that a high, residual drug concentration was present at the times of reinjection. Since the dose is the same and K is practically constant in both experiments, it is evident that the time interval between injections determines the actual maximum concentrations attained.

By fitting the best straight lines to the experimental results, the values for K and C_o in each interval were obtained. A value for C_o can be determined from the data for each injection interval by extrapolation to the time corresponding to the reinjection. Except for the first period where the extrapolation gives C_o directly, subsequent extrapolations for each injection period will result in a value of C_o plus the residual concentration from previous injections. Subtraction of the residual value will give the actual figure for C_o derived from the data of that particular period. In Table IV, the concentrations found experimentally and the values for K and C_o calculated for every interval from the experimental data are recorded. The column headed "calculated" refers to the concentration predicted by substituting, for the first injection interval, the average values of K and C_o in equation (1), and for any subsequent interval, by substituting the appropriate C_n as obtained from the equation (2) for C_o of the equation (1), and

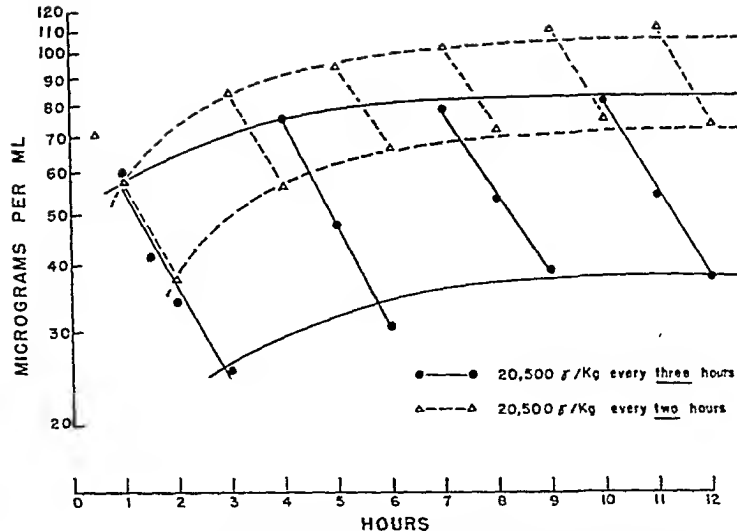


FIG. 3. STREPTOMYCIN CONCENTRATIONS IN THE PLASMA OF DOGS AFTER REPEATED INTRAMUSCULAR INJECTION

TABLE IV

Streptomycin concentrations in the plasma of dogs after repeated intramuscular injections

20,500 γ /KG. EVERY 3 HOURS						20,500 γ /KG. EVERY 2 HOURS					
Time hours	Concentration		Extrapol. conc. at time of injection γ /ml.	C_0 γ /ml.	K hours^{-1}	Time hours	Concentration		Extrapol. conc. at time of injection γ /ml.	C_0 γ /ml.	K hours^{-1}
	Calcd. γ /ml.	Found γ /ml.					Calcd. γ /ml.	Found γ /ml.			
1.0	57.4	60.0	83.8	83.8	-0.180	0.5	69.8	70.5	87.1	87.1	-0.180
1.5	47.2	41.6				1.0	57.4	57.5			
2.0	38.9	34.2				2.0	38.9	38.0			
3.0	26.3	25.5				3.0	83.7	84.5	126.4	88.4	-0.175
4.0	75.2	75.0	118.0	92.5	-0.197	4.0	56.7	56.5			
5.0	50.9	47.7				5.0	95.8	94.5	134.3	77.8	-0.153
6.0	34.5	30.4				6.0	64.8	66.5			
7.0	80.7	78.5	108.7	78.3	-0.150	7.0	101.3	102.0	144.5	78.0	-0.151
8.0	54.7	53.0				8.0	68.6	72.0			
9.0	37.0	39.4				9.0	103.9	110.5	162.8	90.8	-0.168
10.0	82.4	81.0	117.2	77.8	-0.163	10.0	70.3	75.0			
11.0	55.8	54.0				11.0	105.0	110.5	166.1	91.1	-0.177
12.0	37.8	38.0				12.0	71.1	73.5			
Ratio, $r = 0.309$						Ratio, $r = 0.457$					
Max. conc. 1 hr. after inj. = 83.2 γ /ml.						Max. conc. 1 hr. after inj. = 105.9 γ /ml.					
Max. conc. 3 hrs. " " = 38.1 γ /ml.						Max. conc. 2 hrs. " " = 71.7 γ /ml.					

Average values for: $C_0 = 84.8 \pm 6.3 \gamma$ /ml.; $K = -0.170 \pm 0.012$.

considering t as the time measured from the injection at the beginning of the n th interval.

The confining lines in figure 3 are the graphical representation for the predicted range of concentrations with these particular dosing schedules. The maximum concentration indicated on the graph refers to the experimental values 1 hour after injection as obtained from equation (4) and not to the limiting value C_{∞} , derived from the extrapolated value C_0 , through the use of equation (3).

By decreasing the time interval between injections from 3 to 2 hours the total amount of drug injected for the whole period was increased by 50%. After a steady state was established, the rise of the maximum concentration reached one hour after every injection, however, was only 27%, while the minimum concentration was raised by 88%, thereby reducing the spread between the actual maximum and minimum concentration.

Since the values of K and C_0 may be altered by pathological or physiological conditions, the actual maximum can be predicted only when these values are known under a particular set of circumstances. Nevertheless, as long as the rate of decrease in the concentration of the drug in the blood is proportional to the concentration in the blood, repeated injections of the same dose at regular intervals will not build up the drug concentration in the blood beyond a certain maximum for any specific selected dose and injection interval. It should be emphasized that these conditions refer only to concentrations of the drug in the blood, and not to possible accumulation at any other point in the organism.

The authors wish to thank Theodore M. Edison for his suggestions regarding the mathematical treatment of the data for repeated doses.

SUMMARY

Chemical determinations of the concentrations of streptomycin in the blood have been made in dogs and humans, following intramuscular injections of the drug.

The concentrations of streptomycin in the blood were found to be proportional to the dose per kilogram of body weight, within a definite range of dosage. From the results obtained, it is possible to calculate with reasonable accuracy the serum concentrations of the drug in man after intramuscular injection of streptomycin in doses ranging from 4,000 to 20,000 γ /kg.

The rate of decrease of streptomycin concentration was found to be proportional to the concentration of the drug in the blood. The rate constants in man and in the dog were different, while the "apparent" volumes of distribution were similar in the two species.

A study was also made of the plasma concentrations of streptomycin in dogs after repeated injections of the drug at intervals of 2 hours and 3 hours.

A method is presented for the calculation of drug concentration in the blood after repeated injection, applicable to any drug in which the rate of decrease in concentration is proportional to its concentration in the blood. A close correlation was found between the theoretical values thus calculated and the actual values found in dogs after repeated injections of streptomycin at regular intervals.

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PHARMACOLOGICAL STUDIES OF d-TUBOCURARINE AND OTHER CURARE FRACTIONS

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A study of the literature and reviews concerning curare Boehm (1), McIntyre (2) and the recent monograph by McIntyre (3) reveals how incomplete our knowledge is on certain phases of the pharmacology of this drug. Perhaps the greatest weakness has been the reliance and emphasis placed on the extensive data obtained in amphibia, even though many of these investigations date back to the latter part of the 19th century when only crude curare was available. The notorious difference between the reaction of cold blooded animals and mammals in regard to many other drugs has apparently not been sufficient warning to prevent the application of frog data directly to the mammals. This has led to several widely held fallacies in regard to curare action, particularly concerning central effects and excretion.

This report deals with the action of d-tubocurarine, a purified quaternary fraction of curare,¹ Intocostrin (Squibb)² and the dimethyl ether iodide of d-tubocurarine in rats, mice, rabbits, guinea pigs, cats, and dogs with reference to toxicity, excretion, and effects on the cardiovascular and central nervous systems.

The head drop assay and lethal dose. The dropping forward of the head due to the selective paralysis of the neck musculature is referred to as "head drop" (H.D.) and is a useful sign of curarization. The margin between the LD₅₀ and HD₅₀ is the difference in the paralyzing dose for the intercostal and diaphragmatic muscles and the paralyzing dose for the other skeletal muscles, particularly those of the neck. As might be expected, this difference is small and consequently there is a narrow margin between effective curarization and respiratory paralysis.

The rabbit has been found to be an ideal animal for the assay of curare potency. Unless otherwise mentioned 0.05% solutions of the drugs were given intravenously. We have not encountered the wide variations (150%) in head drop dose mentioned by others (4). Further, the prolonged slow injection assay (0.1 cc./15 sec. till H.D.) was found in carefully controlled tests with the same group of 12 animals to be no more accurate than an assay with fast injections (total dose in five seconds). The rabbit should not be unduly excited during the injection as this tends to increase the curarizing action. Reproducible results were consistently obtained by using a single fast injection and this method has been adopted as standard practice for assay of potency. A series of over 250 tests has been conducted

¹ The crystalline d-tubocurarine, quaternary fraction, and dimethyl ether iodide of d-tubocurarine were prepared by Dr. D. L. Tabern of Abbott Laboratories. The quaternary fraction contains all the quaternary bases of curare, including not less than 50% d-tubocurarine.

² Intocostrin contains approximately 60% d-tubocurarine and 40% other quaternary and tertiary bases.

and the data are summarized in Table I. It is expedient to describe the symptoms in the following manner.

A full head drop (H.D.) is one in which the head falls forward and cannot be lifted voluntarily by the animal upon stimulation. A minimum head drop (Min. H.D.) is one in which the head falls forward but is lifted voluntarily when the animal is stimulated.

With head drop doses the degree of limb paralysis varies considerably. It is described as "slight paralysis" when ataxia develops and the animal can barely move but still tends to right itself, and "complete paralysis" when the animal remains in side position and is unable to move the limbs. The curarizing effects begin within two minutes after injection and recovery occurs gradually in a period of 10 to 15 minutes. Often associated with "complete paralysis" is involvement of the respiratory muscles. The intercostal muscles are the first paralyzed usually with a concomitant increase in the amplitude of diaphragmatic con-

TABLE I
Summary d-tubocurarine action in rabbits (i.v. injection)

D-TUBOCURARINE mg./kg. dose	NO. OF RABBITS	EFFECT
0.1	15	2 H.D., 3 Min. H.D., 10 none to ataxia
0.12	5	5 slight paralysis
0.125	67	27 H.D.—2 artificial respiration, 11 Min. H.D., 29 paralysis or ataxia
0.13	6	3 H.D., 1 Min. H.D., 2 ataxia
0.14	9	4 H.D., 2 artificial respiration, 1 Min. H.D., 4 ataxia
0.15	97	67 H.D.—24 artificial respiration, 18 Min. H.D., 12 ataxia
0.16	3	3 H.D.—1 artificial respiration
0.175	45	44 H.D.—27 artificial respiration, 1 Min. H.D.
0.18	5	4 H.D.—3 artificial respiration, 1 Min. H.D.
0.2	13	13 H.D., 11 artificial respiration.

H.D. = Head Drop; Min. H.D. = Minimal Head Drop.

tractions. The latter then gradually decrease and finally cease altogether. When respiration becomes inadequate, tremor and convulsive jerks in the limbs occur. These effects are most probably of anoxic origin since they are not seen when adequate artificial respiration of the positive pressure type is started and continued until both diaphragmatic and intercostal respiratory movements are reestablished.

An examination of Table I shows the progressive increase in the degree of paralysis as the dosage of d-tubocurarine HCl is increased. A significant number of head drops appear at 0.125 mg./kg. and the HD_{50} is approximately 0.15 mg./kg. In the higher dose of 0.175 mg./kg. the number of head drops and complete paralysis increases and more animals require artificial respiration. Because of the clear cut differences observed in the range of 0.125 to 0.175 mg./kg., this range has been utilized in formulating the bioassay for determining curarizing potency of d-tubocurarine. In a series of 10 different d-tubocurarine samples all have given an HD_{50} within the limits of 0.125 and 0.175 and most were at 0.15 mg./kg.

An assay to compare potency was made with samples of d-tubocurarine, a mixture of the quaternary bases of curare, and Intocostrin (Squibb). In making dilutions of the clinical samples it was assumed the Intocostrin contained active principles equal to 3 mg. d-tubocurarine HCl (as the pentahydrate) per cc. For assay the sample is diluted to 0.5 mg. d-tubocurarine/cc. or equivalent. The results are summarized in Table II and indicate the samples are equipotent in curare activity. Likewise the symptoms were indistinguishable among the three preparations. Preliminary experiments with the dimethyl ether iodide of d-

TABLE II

Comparative assay of clinical samples of d-tubocurarine, intocostrin and quaternary base fraction

	NO. OF RABBITS	EFFECT
d-Tubocurarine		
mg./kg. dose		
0.125	4	2 H.D., 1 Min. H.D., 1 slight paralysis
0.15	7	4 H.D., 2 artificial respiration, 2 min. H.D., 1 slight paralysis
0.175	4	4 H.D., 3 artificial respiration
Intocostrin (assumed to contain active principles = 3 mg. d-tubocurarine/cc.)		
0.125	4	1 H.D., 1 Min. H.D., 2 slight paralysis
0.15	7	5 H.D., 2 Min. H. D.
0.175	4	4 H.D., 1 artificial respiration
Quaternary Base Fraction		
0.125	9	3 full H.D. and paralysis, (1 resp. dep. Fatal), 6 no effect
0.15	14	6 H.D., sl. paralysis, (1 art. resp.), 4 min. H. D.
0.175	11	10 full H.D. and paralysis, 5 resp. dep. 2 Fatal, 1 Min. H.D.
0.2	10	10 full H.D., paralysis, resp. dep. 7, 3 Fatal, (no artificial respiration)

tubocurarine reveal that this drug is approximately 10 times as powerful in curarizing properties as d-tubocurarine when tested in rabbits. The head drop dose is 0.015 mg./kg. Except for its greater potency its pharmacological properties appeared identical to d-tubocurarine.

It is well known that crude curare varies greatly in the amount of curarizing principles present. A series of six samples of crude curare, presumably obtained from the Orinoco River region of Venezuela, were assayed by the head drop method using a solution containing 10 mg. of crude curare/cc. The range for the head drop dose was 0.5 to 2 mg./kg., with three samples at 1 mg./kg.

Toxicity in other species. The data obtained in mice are given in Table III.

The onset of ataxia and paralysis occurred within one to two minutes after intravenous injection. No attempt was made to administer artificial respiration and the animals died after typical anoxic jerks and tremors. The LD_{50} lies between 0.1 and 0.125 mg./kg. for mice.

The action of d-tubocurarine in the dog is similar to that observed in rabbits but the effective dosage is greater and somewhat more variable (as shown in Table IV). The curarizing dose lies between 0.15 and 0.25 mg./kg. With the

TABLE III

Toxicity of d-tubocurarine given intravenously in mice (Solution = 0.01 mg. d-tubocurarine/cc.)

DOSE mg./kg.	NO. OF MICE	EFFECT
0.075	10	10 ataxia
0.10	15	7 ataxia, 3 paralysis and resp. dep., 3 Fatal
0.125	15	3 ataxia, 7 paralysis, 4 Fatal
0.15	15	10 complete paralysis, 7 Fatal
0.175	10	10 complete paralysis, 7 Fatal
0.20	10	10 complete paralysis, 10 Fatal

TABLE IV

Action of curare preparations given intravenously in dogs

DRUG	DOSE mg /kg	NO. OF DOGS	EFFECT
d-tubocurarine	0.15	5	2 H.D., 3 sl. paralysis of limbs
	0.2	2	1 H.D., diaphragmatic resp., 1 slight paralysis
Quaternary base fraction	0.15	7	1 H.D., 1 Min. H.D., 4 sl. paralysis of limb, 1 no effect
	0.175	4	3 min. H.D., 1 sl. ataxia
	0.2	10	7 H.D., 1 Min. H.D., 1 ataxia
Intocostin	0.15	2	1 Min. H.D., 1 sl. paralysis of limbs
	0.2	1	1 H.D.

onset of curarization the animal becomes somewhat apprehensive as ataxia develops, and within 2-5 minutes head drop and limb paralysis ensue. Vomiting is sometimes observed and defecation occurred in approximately 50% of the dogs. No signs of excitement or convulsions were observed in any instance, either with Intocostin, the purified quaternary fractions or d-tubocurarine. Recovery from a head drop dose occurred in 10 to 20 minutes.

The results of studies with rats and guinea pigs are shown in Tables V and VI. The drug was injected into the tail vein of rats and the dorsal penile vein of male guinea pigs. The data show that these animals are much more sensitive to

d-tubocurarine than the other species tested. Thus the intravenous head drop dose for rats is 0.075 mg./kg. and for guinea pigs 0.035 mg./kg. These doses also produced respiratory depression. It was noted that guinea pigs showed a gasping reaction as curarization set in. The possibility of a bronchio-constriction was investigated. On autopsy the lungs were generally normal. Furthermore, 3 mg./kg. Pyribenzamine given subcutaneously 10-20 minutes prior to curare did not change the symptoms or the toxicity. This amount of Pyribenzamine protects pigs against thirty times the lethal dose of histamine. Thus histamine release was not demonstrable as a factor in the greater sensitivity of this species.

TABLE V
Effect of intravenous d-tubocurarine in guinea pigs

DOSE <i>mg./kg.</i>	NO. OF PIGS	EFFECT
0.02	1	No effect
0.03	3	1 no effect, 1 sl. ataxia, 1 sl. paralysis
0.035	4	2 F, paralysis and resp. failure, 1 paralysis and resp. dep., 1 sl. paralysis of limbs
0.04	3	3 F, paralysis and resp. failure
0.05	2	2 F, paralysis and resp. failure
0.1	3	Paralysis and resp. failure, fatal
0.2	4	Paralysis and resp. failure, fatal

TABLE VI
Toxicity of d-tubocurarine given intravenously in rats (Solution = 0.025 mg. d-tubocurarine/cc.)

DOSE	NO. OF RATS	EFFECT
0.03	2	1 Min. H.D., 1 slight paralysis
0.05	7	3 H.D., 2 resp. depression, 2 min. H.D., 2 partial paralysis
0.075	9	9 H.D., resp. depression
0.1	9	9 H.D., resp. failure

Careful observation of rats given 0.5 to 2 mg./kg. either intramuscularly or subcutaneously revealed that respiratory rate and amplitude decreased as curarization set in. The resulting anoxia produced violent jerks and tremors of a convulsive type. Administration of artificial respiration with high O₂ resulted in immediate cessation of these anoxic effects. The alleged central stimulating action of curare reported by Cohnberg (5) was almost certainly due to similar anoxic effects and not to the curare per se. Placing rats in an atmosphere of oxygen will not prevent the development of anoxia after the onset of respiratory paralysis. Diffusion of oxygen is inadequate and therefore positive artificial respiration is essential in such experiments.

The effects of large doses of d-tubocurarine were investigated in rabbits and dogs. The chief difficulty encountered was the proper adjustment of artificial

respiration to insure adequate ventilation. Two rabbits given 1 mg./kg. recovered after five hours under artificial respiration. Nine other rabbits given 1.0 and 2.0 mg./kg. of quaternary fractions recovered after one to six hours. One dog given 0.4 mg./kg. recovered in 20 minutes and another given 1 mg./kg. recovered in eight hours. A number of rabbits and dogs given 5 to 10 mg./kg. d-tubocurarine have remained alive under artificial respiration for 24 to 36 hours, but finally succumbed due to pulmonary complications or shock. The dangers of prolonged curarization have been reported previously by Perlstein (6) who found signs of cardiac damage.

The results obtained from intramuscular injections of d-tubocurarine in rabbits are summarized in Table VII. The effective curarizing dose is between 0.3 and 0.4 mg./kg. Artificial respiration was initiated upon the appearance of respiratory depression. In doses of 0.3 mg./kg. or less the elimination of d-tubocurarine is sufficiently rapid to prevent the attainment of an adequate curarizing level. With 0.3 mg./kg., those developing head drop begin to show curarizing effects at

TABLE VII

Action of d-tubocurarine given intramuscularly in rabbits (Solution = 1 mg. d-tubocurarine/cc.)

DOSE D-TUBOCURARINE mg./kg.	NO. OF RABBITS	EFFECT
0.15	5	5 no symptoms
0.2	10	10 no symptoms
0.3	10	2 H.D., 1 min. H.D., 1 partial paralysis, 6 no symptoms
0.4	10	7 H.D.—5 artificial respiration, 1 min. H.D.
0.5	7	7 H.D.—7 artificial respiration

20 to 50 minutes after injection. In doses of 0.4 and 0.5 mg./kg. complete paralysis appears in five to ten minutes and lasts one to three hours.

Pharmacological antagonists to curare. In a series of 20 rabbits the antagonism of d-tubocurarine by neostigmine methyl sulfate was investigated. A dose of 0.05 to 0.1 mg./kg. neostigmine given intravenously produced a definite beneficial increase in respiration in animals previously given 0.2 mg./kg. d-tubocurarine. However neostigmine was ineffective against a dose of 0.3 mg./kg. (just double the head drop dose). If the drug was given prior to the curare, respiration was not paralyzed even with 0.3 mg./kg. d-tubocurarine. Thus it would appear that neostigmine is more effective when given prior to curare and is of limited usefulness as an antagonist since it is ineffective against large doses of d-tubocurarine. This is in accord with the observations of Koppányi (7) and McIntyre (8). In other experiments it was found that 5 to 10 mg./kg. of pentamethylentetrazol was also ineffective as an antidote and did not affect the head drop dose. Metrazol stimulates the respiratory center but has no peripheral action and therefore would hardly be expected to antagonize curare, since the latter does not affect the respiratory center as shown by the experiments of

Harvey (9). He recorded the impulses from the central end of the phrenic nerve by means of an amplifier and found that curare in doses sufficient to paralyze respiration did not affect the respiratory center as reflected by continued phrenic nerve activity. The work by Fegler (10) in which he found that curare depressed respiratory reflexes induced by sciatic stimulation, is complicated by morphine and chloralose anesthesia as well as a complex reflex pathway that could be inhibited at many points other than the respiratory center.

A group of 10 rabbits which had a head drop dose of 0.15 mg./kg. d-tubocurarine were tested again after a rest period of three days. Five minutes prior to d-tubocurarine they were given 1 mg. of atropine intravenously. The symptoms and head drop dose were not altered by atropine.

Metabolic studies and fate of d-tubocurarine. The duration of action of d-tubocurarine was studied in 30 rabbits using a divided dose technique. From the data in Table VI it is seen that the first dose of 0.1 mg./kg. d-tubocurarine causes neither paralysis nor head drop. With 0.2 mg./kg. in one dose, complete paralysis occurs, usually with respiratory depression. Effects greater than could be expected from the second dose of 0.1 mg./kg. alone are observed as long as one hour after the first dose. Therefore some d-tubocurarine must still be present long after all observable signs have disappeared. After two hours no residual additive effects are detectable. Similar experiments with mice showed no residual from the first dose after 20 minutes.

In a series of Warburg studies using thinly sliced cerebral cortex of mice, d-tubocurarine in doses of 1 and 2 mg. added to the Ringer-glucose (total volume 3 cc.) had no effect on oxygen consumption. This is in accord with the findings of Featherstone and Gross (11) for rat brain mince using pyruvate as substrate.

The fate of curare in the body has been generally accepted to involve partial destruction in the liver and excretion by the kidneys (1). It was surprising to find therefore that in a group of eight double nephrectomized rats there was no increase in sensitivity to or duration of action of d-tubocurarine given intravenously. Likewise in nine hepatectomized rats (70-90% of liver removed) no increase in duration of action was noted. In controls, sham operated, and operated rats, 0.075 mg./kg. was the paralytic dose and the duration of paralysis was approximately five minutes. Another group of eight rats with both double nephrectomy and hepatectomy also showed no prolongation of curare action. In the first two groups the drug was given 24 hours after operation. In the double operation group the test was made 4-6 hours after operation as the animals die within 12 hours after this procedure. That the liver is of secondary importance in detoxifying curare is also indicated by work in Eck fistula dogs (1). The present studies do not imply that the kidney and liver may not play a part in excretion or metabolism of d-tubocurarine but rather indicate that these mammals can detoxify curare without kidney or liver function. This was further supported by the observation that upon injection of the second curare dose two hours after the first dose there was no indications of residual curare from the first injection.

Similar studies in 10 nephrectomized rabbits also showed no increased sensi-

tivity to head drop doses of curare. Duration of paralysis was five to fifteen minutes as in normals. A further series of nephrectomized rats and rabbits were given d-tubocurarine intramuscularly. In this group the sensitivity of the nephrectomized animals was slightly greater than in sham operated controls. Thus the nephrectomized rabbits had full paralysis and respiratory depression on 0.3 mg./kg. I.M. while controls had only head drop and partial paralysis. Duration of action was not significantly changed however.

Effect of d-tubocurarine on the cardiovascular system. In the frog heart (Straub preparation) cardiotoxic effects are not evident with less than 1 mg. of d-tubocurarine in 1 cc. of Ringer Solution. In doses of 1 to 4 mg. partial heart block occurs, usually a 2-1 auricular-ventricular block. The amplitude is also decreased. The heart is arrested in diastole by 5 mg. This condition is reversible after washing. Considering the minute amount of tissue involved it is obvious that d-tubocurarine has very little cardiotoxic action. Although no accurate estimate can be made, the cardiotoxic level must be of the order of 1000 times the curarizing dose. Studies with Langendorf preparations of the rabbit heart using blood-Ringer for perfusion fluid gave similar results. Injection of 1 mg. d-tubocurarine in 2 cc. of Ringer into the canula entering the heart did not affect the rate of contraction.

The action of d-tubocurarine on blood pressure was investigated in six cats and two dogs under phenobarbital-pentobarbital anesthesia. The results were essentially similar in all experiments. Doses of 0.1 to 0.2 mg./kg. caused small drops in blood pressure (10-20 mm.) while with large doses (0.4 to 1 mg./kg.) which produced respiratory paralysis, a more profound fall of blood pressure of 50 to 70 mm. occurred. There was usually little or no recovery, the blood pressure remaining at 50 to 80 mm. One mg./kg. of atropine prior to d-tubocurarine had no effect on the response. The low blood pressure level was maintained for several hours and further doses of 1 to 10 mg./kg. d-tubocurarine caused only slight transitory depression. Similar results were observed with Intocostin and the quaternary fraction. It was also noted that the dose of curare necessary to produce respiratory paralysis was approximately the same as for normal animals and was not affected by the deep pentobarbital-phenobarbital anesthesia used in these blood pressure studies. The fall in blood pressure is most probably due to peripheral effects with poor venous return resulting from the complete loss in muscle tone, lack of diaphragmatic movement, and dilation of peripheral blood vessels as contributing factors. Injection of ephedrine or methyl-iso-thiourea (0.5 to 1 mg./kg.) in most instances produced some increase in blood pressure lasting for 10 to 30 minutes. Epinephrine was most reliable and always caused a transitory rise in blood pressure. Similar observations have been made by Cole (12) who found ephedrine would partially restore the blood pressure in dogs which had been given large doses of Intocostin. Here too epinephrine was a more reliable vasopressor.

One mg./kg. Pyribenzamine prior to d-tubocurarine in anesthetized cats decreased the transitory drops in blood pressure which follow immediately after curare injection. This transitory fall may thus be due in part to a release of

histamine by curare as indicated by the work of Comroe (13). The overall gradual decline in blood pressure with increasing doses of curare is not prevented however. The low blood pressure and the engorgement and hemorrhage found in the small intestine of dogs is considered by Cole (12) to be indicative of shock from large doses of curare.

In three cats the response of blood pressure to acetylcholine, histamine and epinephrine was tested before and after giving 0.2 mg./kg. d-tubocurarine. Doses of histamine and acetylcholine were chosen which gave drops of 20 to 30 mm. and the epinephrine injection a rise of 30 to 40 mm. Hg. After 0.2 mg./kg. curare the blood pressure fell 20 mm. At this new base level, responses to histamine and acetylcholine were unchanged and the epinephrine effect was somewhat prolonged.

In an unanesthetized 18 kg. dog a study using the Hamilton manometer revealed that a paralytic dose of d-tubocurarine (0.2 mg./kg.) caused only a transitory fall in blood pressure of 20 mm. with little change in heart rate.

Effect of d-tubocurarine on the EKG. These studies were made on unanesthetized rabbits, dogs, and cats, using the standard leads. A control record was taken before giving d-tubocurarine. Artificial respiration, with an oxygen stream added in some instances, was given at the first signs of respiratory paralysis.

In five rabbits doses of 0.2 to 10 mg./kg. d-tubocurarine were given intravenously. Even with the large doses very little effect on the heart was observed. The rate slowed by approximately 10%, and the EKG showed little or no change. A high T wave and slowing appeared when artificial respiration was inadequate.

Similar studies were made in three dogs. The heart rates varied from 130 to 190 and did not decrease after injection of 0.3 mg./kg. d-tubocurarine. With further doses up to 10 mg./kg. the record showed an increased T in lead II but no changes in the EKG indicative of damage to cardiac conduction were observed.

Further studies were made in unanesthetized cats. Heart rates, EKG, and blood pressure were recorded. Doses of d-tubocurarine from 0.2 mg. to 5 mg./kg. were injected intravenously. There was no significant change in heart rate. Blood pressure fell from 150 to 90 mm. with doses of 1 mg./kg. Similar records were obtained in cats given 1 mg./kg. atropine prior to curare.

Histamine-like effects. The response to d-tubocurarine and Intocostrin observed in the isolated perfused rabbits ear upon the introduction of 1 to 5 mg. d-tubocurarine into the perfusion fluid was a 50 to 80 per cent decrease in the rate of flow indicating vasoconstriction. Histamine likewise caused a marked decrease in perfusion rate. According to Dale the arterioles of rodents are constricted by histamine, and there is considerable evidence that curare releases histamine (Comroe and Dripps (13)), which might account for these observations. Opposite effects might be expected in other species where dilation of arterioles by histamine is the rule. In support of possible histamine release by curare were observations made on perfused guinea pigs lungs. With dose of 0.5 mg./kg. no effect was observed but the addition of 1 mg. d-tubocurarine caused a decrease in rate of flow of 25 to 40%. This bronchio-constriction was antagonized by

epinephrine. As previously mentioned no bronchio-constriction was demonstrable with paralytic doses given *in vivo*.

Effect of d-tubocurarine on the intestine. The effect of d-tubocurarine on isolated segments of rabbit intestine was investigated. In doses of 0.25 to 0.5 mg. of d-tubocurarine added to a 20 cc. Ringer bath, a consistent increase (20%) in amplitude was observed. According to Grass and Cullen (14) higher doses cause inhibition of the gut, and their *in vivo* studies using unanesthetized Tbirdy-Vella fistula dogs showed a consistent temporary loss of tone and peristaltic activity of the small intestine with therapeutic doses of Intocostin and d-tubocurarine. As mentioned previously, half of our dogs defecated after injection of curare which would seem to indicate at least a transient stimulation of the colon.

Local anesthetic test. In guinea pigs intracutaneous wheals of d-tubocurarine solution (3 mg./cc.) were stimulated electrically. No anesthetic action was observed over a period of five minutes. There was an erythemic reaction causing the wheal to redden, probably due to histamine release. In five minutes paralysis and respiratory failure occurred due to the absorbed curare. Likewise no topical anesthetic action was found after instillation of d-tubocurarine into eyes of rabbits.

The effects of d-tubocurarine on the central nervous system. A preliminary report of this work has appeared (15). In a series of studies on unanesthetized cats, rabbits, and rats either prepared with implanted epidural electrodes or silver electrodes on exposed cortex, the effect of d-tubocurarine on the EEG, the spike response to stimulation of contralateral cortex, and the induction of convulsive activity by electro-shock or pentamethylentetrazol were determined. Great care is necessary in such studies to insure adequate artificial respiration. Usually a stream of O₂ was added. No signs of convulsions or twitching were observed unless anoxia was allowed to develop. The curarizing dose sufficient to cause respiratory paralysis in all these species is approximately 0.2 mg./kg. d-tubocurarine. With doses from five to 50 times this amount no effect on the EEG was noted. It was further found that the normal convulsive dose of Metrazol produced a typical firing of the cortex although all motor manifestations were absent and the electromyograph showed no spikes. Likewise no change in electroshock threshold was observed. Direct stimulation of the muscles caused vigorous contractions.

In a series of rabbits d-tubocurarine was injected directly into the carotid artery which had been exposed previously using procaine in the area of operation. Even with the rapid injection of 1 mg./kg. d-tubocurarine no convulsive movements were observed. Paralysis and respiratory failure occurred within a minute. These results stand in contrast to observations made in frogs where intra-aortic injection of curare caused convulsions (16). However in this early work crude curare was used.

Our results are in disagreement with the work of Pick and Unna (18) in frogs and the studies of McIntyre (19) in anesthetized dogs. These investigators observed a depression of the EEG by curare. There are considerable difficulties in interpreting the drug effects on the EEG in the presence of an anesthetic

agent since the latter usually produces signs of depression. Furthermore the studies in unanesthetized dogs by Smith *et al.* (20) and Toman (21) and in cats and monkeys by McCulloch (22) confirm our observations that little or no central depression is demonstrable in curarized animals.

In contrast are the marked central stimulating effects obtained when d-tubocurarine is injected intracisternally or into the cerebral cortex. In doses of 0.1 mg. to 0.5 mg./kg. given intracisternally to rabbits, violent tonic-clonic convulsions appeared in one to three minutes and continued for over an hour. Most animals died eventually from curarization. Similar effects have been observed with crude curare and curarine (1). This action of curare is comparable to that observed with penicillin which also has no central effects when given intravenously but is a convulsant when applied directly to the CNS (17).

Discussion. The most impressive property of curare is its highly specific paralytic action at the neuromyal junction. Its effect on other organs is relatively slight even with doses greater than the effective paralytic dose. Other than the respiratory depression resulting from muscular paralysis the most marked effect is the fall in blood pressure. With high doses the vasodepressor action may be very great producing a shock like condition. Vasopressor drugs are able partially to restore blood pressure, which would tend to indicate that the fall in blood pressure is due to vasodilatation as well as decrease in muscle tone and loss of diaphragmatic movements.

The experiments showing the ability of mammals to detoxify just paralytic doses of curare in the absence of either the kidneys or liver or both, stand in sharp contrast to the older observations on amphibia (1) and indicate again the dangers of applying data obtained in amphibia to the mammals. A significant factor in making such comparison would also involve the consideration of dosage, since the dose of curare used in the amphibia is much greater than used in the mammals. It is not uncommon to find that therapeutic doses of a drug may be handled by the body in the absence of the kidneys or liver but that larger toxic doses cannot be coped with.

We have observed that d-tubocurarine has little or no action on the higher centers of the CNS when given intravenously to rats, rabbits and cats. However when this drug is injected intracisternally directly into the cerebro-spinal fluid, convulsions are produced even with small doses. This difference in effect would seem to indicate a blood brain barrier in regard to the passage of d-tubocurarine into the central nervous system. The interpretation of the records shown in the paper by McIntyre indicating a depression of activity in the electroencephalogram is complicated by the use of a barbiturate, and possibly anoxic effects. Further the alleged convulsant property of Intocostrin and d-tubocurarine reported by Cohnberg has been shown in similar experiments to arise from inadequate respiration resulting in anoxic tremors and convulsions. In small animals, where oxygen consumption is high and respiratory rate is rapid a decrease in rate and depth of respiratory movements may result in anoxic effects before profound muscular paralysis is evident.

Clinical implications. The results from the experiments reported here may

have certain possible clinical implications with regard to overdosage. The experiences with artificial respiration point to the importance of establishing an adequate airway and the use of positive pressure artificial respiration. The addition of oxygen until anoxic signs have disappeared is recommended. With just paralytic doses blood pressure effects are transitory, however with large doses of curare a vasomotor collapse and shock-like condition may occur. During the first stages of respiratory paralysis the blood pressure may increase due to increased CO_2 . After adequate ventilation is obtained the blood pressure falls to subnormal levels. The use of long acting vasopressor drugs in such cases would seem indicated. The results reported by Koppanyi (7) and by McIntyre (8) in studying antidotes for curare confirm this point of view. Our studies further substantiate the ineffectiveness of neostigmine as an antidote against large doses of curare. The present experiments on the central nervous system and the clinical study by Smith *et al.* (20) support the view that d-tubocurarine has little or no effect on this system even though large doses have been given intravenously or intramuscularly and thus the use of either central stimulants or depressants in curare poisoning is contraindicated. The introduction of curare preparations into the cerebrospinal fluid or otherwise brought into direct contact with the central nervous system will produce central excitation and convulsions. Thus curare should be used with caution in cases where damage to blood vessels might allow the drug direct access to the brain or cord. The present studies on the metabolism of curare in mammals stand in contrast to earlier observations made primarily in cold blooded animals and suggest that curare in therapeutic amounts may be destroyed by the body in the absence of either the kidneys or the liver. Thus the use of curare in cases of kidney and liver damage is not necessarily contraindicated. This is supported by clinical evidence (23). Some of the histamine-like reactions which have been observed with curare may be controllable by the use of the new antihistaminic drugs. This remains open for clinical investigation.

The author wishes to acknowledge the cooperation and technical assistance of A. H. Smith, Jr. in these studies.

SUMMARY

1. The head drop assay for curare potency is described in detail. The head drop dose falls within the range of 0.125 to 0.175 mg./kg. d-tubocurarine for the rabbit.
2. The toxicity in other species indicates that rats and guinea pigs are more sensitive, having an HD_{50} of 0.075 and 0.035 mg/kg. respectively.
3. Neostigmine is a limited antagonist of curare since it is effective against just paralytic doses of d-tubocurarine but is ineffective against higher doses.
4. No change from normal in the degree of paralysis or duration of action of d-tubocurarine was noted in nephrectomized or hepatectomized rats and rabbits. This supports the view that the rest of the body can cope with paralytic doses of d-tubocurarine.

5. With minimum paralytic doses there is no change in blood pressure. As respiration fails the blood pressure falls. High doses cause a vasomotor collapse similar to shock; pressor drugs are partly effective against this vaso-depression.

6. EKG records of cats, dogs, and rats indicate no marked change in rate or in the P-QRST complex with large doses of d-tubocurarine. The relatively low cardiotoxic properties of d-tubocurarine were also ascertained in Straub and Langendorf heart preparations.

7. The EEG in unanesthetized rats, rabbits, and cats remained unchanged with 5 to 50 times the paralytic dose of curare given IV. No signs of central stimulation or depression were seen.

8. When curare is given intracisternally to rabbits it produces violent convulsions with doses of 0.1 mg./kg.

9. The importance of adequate positive artificial respiration preferably with the addition of oxygen, to eliminate effects due to anoxia is emphasized.

10. The possible clinical implications of these studies are discussed.

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PHARMACOLOGICAL PROPERTIES OF A NEW ANTIHISTAMINIC, 2-METHYL-9-PHENYL-2,3,4,9-TETRAHYDRO-1-PYRIDINDENE (THEPHORIN¹) AND DERIVATIVES²

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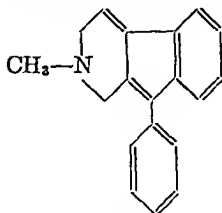
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The accumulating evidence on the important role of histamine in anaphylactic shock and allergic conditions has in recent years stimulated the search for specific histamine antagonists. The attempts which have been made to synthesize such substances have led to a number of quite potent compounds (1-7). Most of these substances can be considered as derived from thymoxy-ethyl-diethylamine (929 F) and N-phenyl-N-ethyl-N'-diethylethylenediamine (1571 F). The former and β -dimethylaminoethyl benzhydrol ether (Benadryl) have an ether linkage in common and the latter was the first of a series of ethylene-diamine derivatives. Reviews on ethylene-diamine derivatives (8) and on anti-histaminics in general (9) have recently been published.

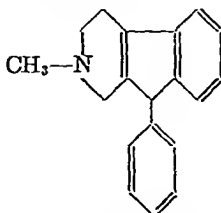
A class of compounds which is chemically unrelated to known anti-histaminics has been synthesized in these Laboratories (10). These are derivatives of pyridindene (indenopyridine) which contain the following ring system.



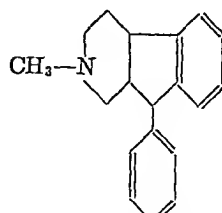
Among a number of such compounds the 2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene (Thephorin) showed potent anti-histamine activity. This paper^r is limited to the pharmacological characterization of Thephorin and of two closely related compounds which differ in the number of the double-bonds, 2-methyl-9-phenyl-2,3-dihydro-1-pyridindene (Nu-1326) and 2-methyl-9-phenyl-2,3,4,4a,9,9a-hexahydro-1-pyridindene (Nu-1525).



Nu-1326



Thephorin
Nu-1504



Nu-1525

¹ T.M. Reg. U. S. Pat. Off.

² The compounds were synthesized by Drs. W. Wenner and J. T. Plati of Hoffmann-La Roche, Inc.

Nu-1326 and Nu-1525 were used as the hydrobromides, Nu-1504 as the hydrochloride or the hydrogen tartrate. Both salts of Nu-1504 are colorless, crystalline substances which are soluble in water. They are equally potent if used in equimolecular amounts. All doses of Nu-1504 are expressed as the hydrochloride regardless of the kind of salt used to allow a fair comparison with known antihistaminics which were available only as hydrochlorides.

ACTION ON THE BRONCHIOLES. *Protection against histamine spray.* The technique employed was essentially the same as described by Loew et al. (3). An air pressure of 120 mm. Hg was used to vaporize about 3 cc. per minute of a 0.5% histamine phosphate solution resulting in a concentration of 0.4 mg. histamine base per liter air. The experiments were carried out with guinea pigs weighing from 200–360 g., in two ways, as follows:

1) The animals were exposed individually to the spray before and 30 minutes after intraperitoneal injection of the substances to be tested, and the time re-

TABLE 1

Protection of guinea pigs against convulsions when exposed to histamine spray for 6 minutes
No. of protected animals/no. of animals used

MG./KG. I.P., 30' PRIOR TO EXPOSURE	THEPHORIN Nu-1504	Nu-1326	Nu-1525	BENADRYL	AMINO- PHYLLINE	EPINEPHRINE
50.0					4/6 (66)*	
30.0			2/15 (13)			
12.0		6/6 (100)				
6.0		2/6 (33)				
4.0				13/15 (87)		
2.0	11/12 (92)			1/5 (20)		
1.0	4/6 (66)					
0.5						4/6 (66)

* Per cent protected.

quired to produce convulsions was recorded. The guinea pigs were removed from the chamber immediately after the onset of convulsions. Those animals which had no convulsions after exposure for 6 minutes were arbitrarily called "protected". It takes from 50–90 seconds to produce convulsions under these experimental conditions. The data are compiled in table 1. 2 mg./kg. Thephorin protect the majority of the animals whereas twice as much Benadryl is required to obtain a similar response. Of the two other pyridindene derivatives, Nu-1326 and Nu-1525, only the former shows some anti-histamine activity. The broncho-dilators, epinephrine and aminophylline exhibit anti-histamine action under these experimental conditions also, as previously shown by Loew (3).

2) 30 minutes after the guinea pigs had received intraperitoneally the anti-histaminic substance, they were exposed in groups of four to the histamine spray for 10 minutes. The number of survivors are recorded in table 2. A total of 52 non-pretreated animals served as controls. The doses of the various com-

pounds required to protect 50% of the animals (PD50) arrived at by interpolation are: 0.42 mg./kg. for Thephorin, 0.8 mg. for Benadryl and <0.25 mg./kg. for Pyribenzamine. Thus the approximate relative potencies derived from this test are: Thephorin:Benadryl:Pyribenzamine = $1\frac{1}{2}$:>2. The safety margin in guinea pigs as calculated from the PD50 and LD50 (see table 7) is $\frac{140}{0.42} = 333$.

Protection against intracardially injected histamine. Thephorin is also quite effective in protecting guinea pigs against death from intracardially injected histamine. The volume of the histamine phosphate solution was kept constant at 1.0 cc./kg., regardless of the absolute histamine dose. The guinea pigs used weighed from 200–350 g. As table 3 demonstrates, 5 mg./kg. of Thephorin intraperitoneally protected the animals against 15 median lethal doses of histamine. The corresponding figures for equal amounts of Benadryl and Pyriben-

TABLE 2

Protection of guinea pigs against death, exposed for 10 minutes to histamine spray 80 minutes after injection of anti-histaminics

No. of protected animals/no. of animals used

MG./KG., I.P.	CONTROL	THEPHORIN Nu-1504	NU-1326	NU-1525	BENADRYL	PYRIBENZAMINE
—	1/52 (2)*					
30.0				0/20 (0)		
12.0			15/30 (50)			
6.0			1/10 (10)			
4.0		40/40 (100)			26/32 (81)	
2.0		19/25 (76)			16/24 (67)	
1.0		21/25 (84)			15/25 (60)	
0.5		14/25 (58)			7/25 (28)	19/20 (95)
0.25		5/20 (25)				13/20 (65)

* Per cent protected.

zamine were 7 and 30, respectively. Hence the relative potencies of these three compounds are in good agreement with those obtained from the spray test. The relatively long lasting affect of Thephorin is striking. Marked anti-histamine action is still evident 5 hours after Thephorin administration.

Whereas the broneho-dilators, epinephrine and aminophylline, were quite active in the spray test, they were ineffective against intracardial histamine. All twenty guinea pigs used died from the intracardial injection of only 3 median lethal doses although they had received intraperitoneally 0.5 mg./kg. or 1.0 mg./kg. epinephrine 10 minutes prior to the histamine injection. Similarly, none of ten guinea pigs pretreated intraperitoneally with 50 mg./kg. aminophylline survived when they received intracardially 3 median lethal doses of histamine 30 minutes later.

We investigated the relationship between various doses of histamine and the amount of Thephorin necessary to protect 50% of the guinea pigs used. From the results which are presented in table 4, a linear-relationship can be assumed.

TABLE 3

Protection of guinea pigs against death from intracardially injected histamine
No. of protected animals/no. of animals used

HISTAMINE BASE, MG/KG, 30' AFTER DRUGS	NO OF MED LETHAL DOSES (LD50)	CONTROL	THEPHORIN 5 MG/KG. I.P.	BENADRYL 5 MG/KG. I.P.	PYRIBENZAMINE 5 MG/KG. I.P.
.2		8/10 (80)*			
.3		4/10 (40)			
.4	1.5	0/10 (0)			
1.2	4.5			6/10 (60)	
2.0	7.5		13/13 (100)	3/10 (30)	
2.4	9.0		13/20 (65)		
4.0	15.0		8/20 (40)		7/10 (70)
6.0	22.5				3/10 (30)
8.0	30.0				4/10 (40)
2½ hours after drug					
1.2	4.5		10/15 (67)		
2.0	7.5		0/5 (0)		
5 hours after drug					
1.2	4.5		9/20 (45)		

* Per cent protected.

TABLE 4

Relation of intracardial histamine toxicity in guinea pigs to the intraperitoneal dose of Thephorin

THEPHORIN I.P., MG/KG.	Histamine base i.c. 30' after Thephorin			NO OF PROTECTED ANIMALS/NO. OF ANIMALS USED	% PROTECTION
	MG/KG.	NUMBER OF MEDIAN LETHAL DOSES	LD50 ± S.E., MG/KG		
0	0.2			8/10	80
0	0.3			4/10	40
0	0.4	1.5		0/10	0
1.25	0.8	3		7/10	70
1.25	1.2	4.5	1.08 ± 0.26	4/12	33
1.25	2.0	7.5		1/8	12
2.5	1.2	4.5		10/10	100
2.5	2.0	7.5	2.25 ± 0.45	6/10	60
2.5	2.4	9		4/10	40
5.0	2.4	9		13/20	65
			3.3 ± 0.75		
5.0	4.0	15		8/20	40
10.0	8.0	30		13/20	65
10.0	12.0	45	9.6 ± 1.6	6/20	30
10.0	16.0	60		3/20	15
20.0	12.0	45		14/20	70
			18.0 ± 5.0		
20.0	24.0	90		7/20	35
40.0	24.0	90		15/20	75
40.0	32.0	120	31.0 ± 4.4	9/20	45
40.0	40.0	150		0/10	0

The line in fig. 1 is drawn through calculated points using the method of the least squares ($y = .56 + .77 x$). It fits the experimentally obtained points fairly well except for the one which corresponds to 40 mg./kg. Thephorin. At this dose ($\frac{1}{3}$ of LD50) some of the animals showed toxic symptoms which may account for the marked deviation of this point.

PROTECTION AGAINST ANAPHYLACTIC SHOCK. Guinea pigs actively sensitized by the intraperitoneal injection of 2 cc. of a 10% egg white solution received intracardially 0.5 cc. of a 2% solution as shocking dose 18 days later. Thephorin seems to be somewhat more protective than Benadryl. The potency difference, however, is not as definite as it is against injected histamine, and in addition, as

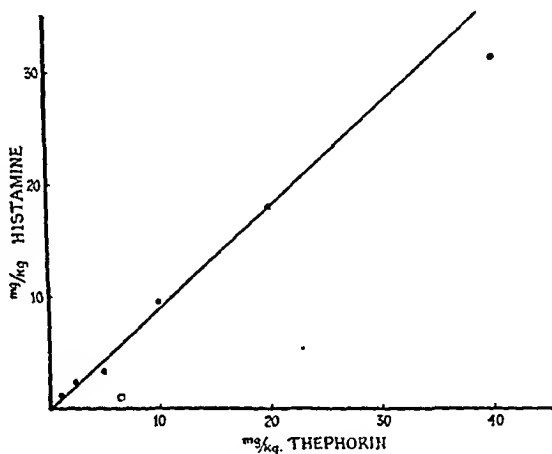


FIG. 1. RELATION OF INTRACARDIAL HISTAMINE TOXICITY IN GUINEA PIGS TO INTRAPERITONEAL DOSE OF THEPHORIN

Abscissa: mg./kg. Thephorin i.p. Ordinate: mg./kg. Histamine base

has been shown previously for another compound (11), protection by both Thephorin and Benadryl is less perfect against anaphylactic shock than it is against histamine shock.

PROTECTION AGAINST BRONCHOCONSTRICTION FROM HISTAMINE IN CATS. The anti-histamine action of Thephorin can also be demonstrated on the bronchioles of the cat "in situ", recorded as by Konzett and Rössler (12). A typical record is seen in fig. 2. Similar results were obtained from experiments on six cats in deep dial-urethane anesthesia. Large doses of histamine are required in this species to produce marked bronchoconstriction. Acetyl-choline induced bronchospasm is less pronounced and more transient. The spasmogenic effects of both agents are prevented by a previous intravenous injection of 1 mg./kg. Thephorin.

ANTISPASMODIC ACTION ON ISOLATED ORGANS. Antispasmodic activity was determined on isolated ileal strips of rabbits and guinea pigs as described in a previous paper (13). Acetyl-choline, Ba-ions and histamine which served as spasmogenic agents, were added to the tissue bath prior to the antispasmodics.

The relative potencies of the various compounds tested are presented in table 5. Thephorin and Benadryl are equally potent against acetyl-choline induced

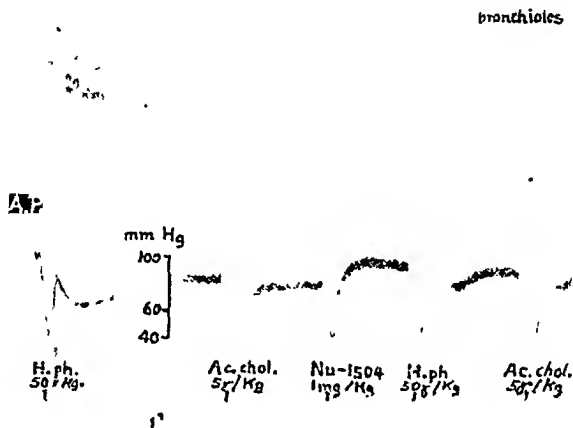


FIG. 2. ANTAGONISM OF THEPHORIN (NU-1504) AGAINST HISTAMINE INDUCED BRONCHOSPASM

Cat 3.0 kg., deep dial-urethane anesthesia. From the top down, bronchioles, carotid arterial pressure signal of injection, time in minutes. All injections through cannula into femoral vein washed in with 1 cc. saline. From left to right, per kg., histamine phosphate 50 micrograms, acetyl-choline bromide 5 micrograms, Thephorin (Nu-1504) 1 mg., histamine phosphate 50 micrograms, acetyl-choline bromide 5 micrograms.

TABLE 5

Relative antispasmodic potency on isolated intestinal strips
Unity is given arbitrarily to Thephorin

	AGAINST		
	Histamine phosphate (guinea pigs) 2×10^{-7} gms/cc.; Tyrode, 30°C	Acetyl choline Br (rabbit) 10^{-3} gms/cc.; Locke, 37°C	BaCl ₂ (rabbit) 2×10^{-4} gms./cc.; Locke, 37°C.
Nu-1504 (Thephorin)	1	1	1
Nu-1326	1/60	<1/10	1
Nu-1525.	1/150	1/5	1/2
Benadryl	1/2	1	1
Pyribenzamine	3	1/5	1/2
Atropine		90	
Papaverine			1

spasm, and both are considerably more active in this respect than Pyribenzamine. Thephorin is also more potent than its congeners Nu-1326 and Nu-1525. There is little difference in anti-Barium action. The relative potencies against

histamine are in good agreement with those obtained from the various "in vivo" experiments.

In determining anti-histamine potency on the isolated guinea pig's intestine, the time elapsed between the addition to the bath of antispasmodic and spasm-

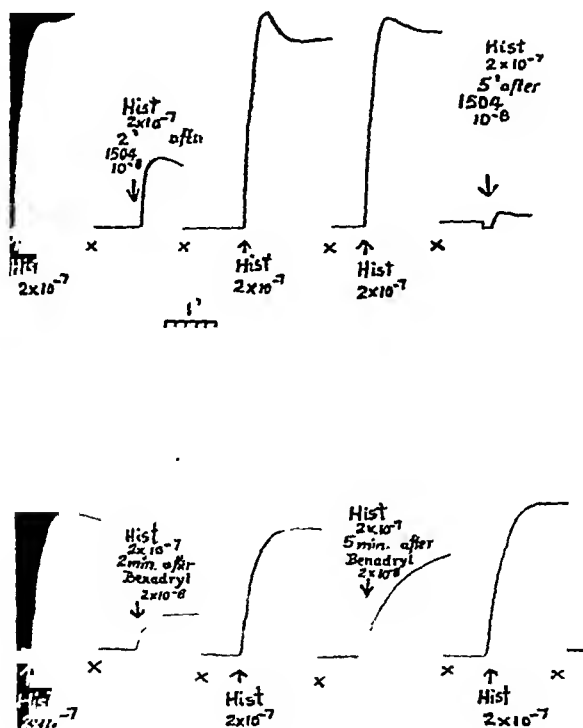


FIG. 3. HISTAMINE ANTAGONISM OF THEPHORIN (Nu-1504) AND BENADRYL ON ISOLATED GUINEA PIG'S INTESTINE IN TYRODE AT 30°C.

Time in minutes. At X washout. At ↑ control administrations of histamine phosphate 2×10^{-7} gms./cc. 2 minutes and 5 minutes after Thephorin (Nu-1504) 10^{-8} gms./cc. Lower record: at ↓ from left to right, histamine phosphate 2×10^{-7} gms./cc. 2 minutes and 5 minutes after Benadryl 2×10^{-8} gms./cc.

genic is an important factor if the agents are given in this order, as illustrated by fig. 3. With increasing time interval the effect of Thephorin becomes more pronounced whereas the reverse is true of Benadryl. The latter can be washed out with ease whereas frequent changes of the bath fluid are required to remove Thephorin from the intestinal tissue.

In addition, Thephorin abolishes spasm from adrenaline of the isolated non-

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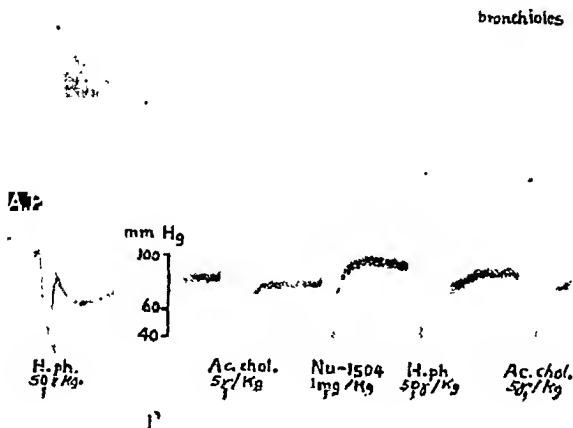


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Nu-1326.	1/60	<1/10	1
Nu-1525..	1/150	1/5	1/2
Benadryl	1/2	1	1
Pyribenzamine	3	1/5	1/2
Atropine.		90	
Papaverine.			1

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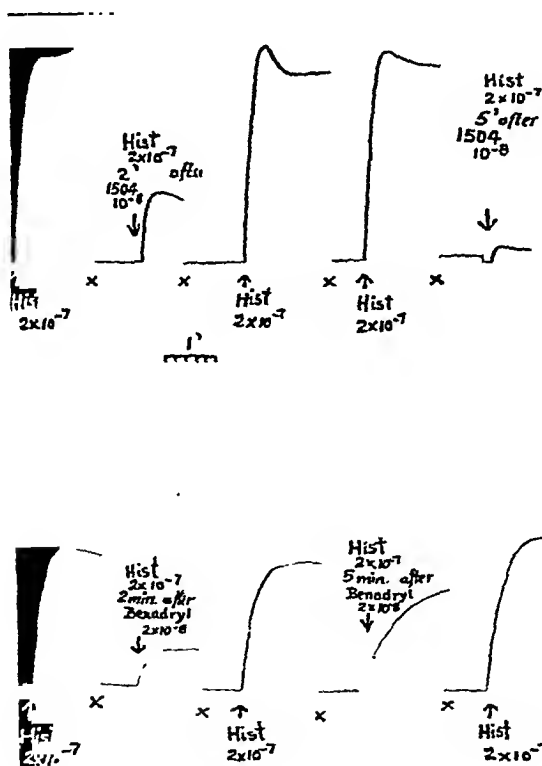


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Time in minutes. At X washout. At ↑ control administrations of histamine phosphate 2×10^{-7} gms./cc. Upper record: at ↓ from left to right, histamine phosphate 2×10^{-7} gms./cc. 2 minutes and 5 minutes after Thephorin (Nu-1504) 10^{-8} gms./cc. Lower record: at ↓ from left to right, histamine phosphate 2×10^{-7} gms./cc. 2 minutes and 5 minutes after Benadryl 2×10^{-8} gms./cc.

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In addition, Thephorin abolishes spasm from adrenaline of the isolated non-

pregnant rabbit's uterus, whereas even double the concentration of Benadryl is considerably less effective in this respect as seen in fig. 4. The difficulty of removing Thephorin by washing is also observed on the isolated uterus. Spasm of the guinea pig's uterus produced by 2×10^{-7} g./cc. of histamine phosphate is completely abolished by 10^{-7} g./cc. of Thephorin. Spontaneous motility of uteri taken from rabbits after several days' treatment with stilbestrol is annulled by 2×10^{-5} g./cc. Thephorin. It has previously been shown (14) that anti-

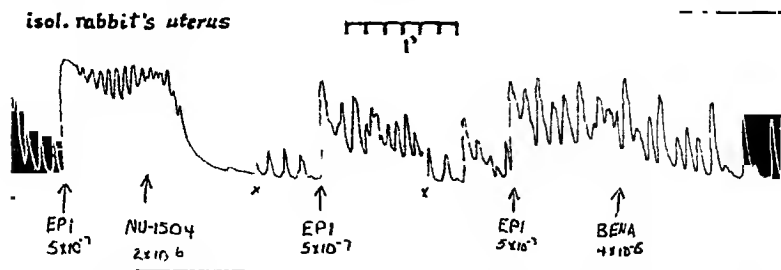


FIG. 4. EPINEPHRINE ANTAGONISM OF THEPHORIN (NU-1504) AND BENADRYL ON ISOLATED RABBIT'S UTERUS

From left to right in gms./cc., epinephrine HCl, 5×10^{-7} ; Nu-1504, 2×10^{-6} ; epinephrine HCl, 5×10^{-7} ; epinephrine HCl, 5×10^{-7} ; Benadryl, 4×10^{-5} .

TABLE 6

Local anesthetic action of thephorin and analogs

	DURATION IN MINUTES*			
	1 %	05 %	.025 %	0125 %
Procaine	45	30	20	15
Thephorin	70	65	25	20
Nu-1326 ..	50	45	25	15
Nu-1525	40	30	20	20

* Figures represent average values from 3 guinea pigs for each compound and each concentration.

spasmodies which lack strong anti-histamine activity often produce spasm of the rabbit's uterus.

LOCAL ANESTHETIC ACTION. The three pyridindene derivatives are fairly potent local anesthetics. On the rabbit's cornea, 1% solutions of Thephorin, Nu-1326 and Nu-1525 produce deep anesthesia lasting for 29, 21, and 37 minutes respectively. These figures represent average values from six rabbit's eyes each.

Using the intracutaneous wheal test in guinea pigs (15), Thephorin and its analogs were found to be longer acting local anesthetics than procain hydrochloride (table 6).

TOXICITY. Acute toxicity of Thephorin as the hydrochloride was determined in mice, rats, guinea pigs, rabbits and dogs. The median lethal doses and the

standard errors calculated according to Bliss (16) are presented in table 7. Thephorin is about as toxic as Benadryl. Fatal doses cause severe tonic-clonic convulsions followed by paralysis and cessation of respiration.

Chronic toxicity studies on oral administration have been carried out in rats and dogs up to eight months without encountering untoward symptoms. Daily doses as high as 50 mg./kg. in rats and 30 mg./kg. in dogs were used. Blood analyses were normal and histological tissue examination did not reveal any pathological changes

TABLE 7
Acute toxicity
LD₅₀, mg /kg \pm S.E

ROUTE OF ADMINISTRATION	THEPHORIN No 1504	No 1326	No 1525	BENADRYL	PYRIBENZAMINE
In mice					
Intraperitoneal	(30)* 88 \pm 11	(30) 150 \pm 18	(25) 110 \pm 14	(20) 85 \pm 14	(26) 83 \pm 14
Intravenous	(20) 22.5 \pm 2.5	(20) 40 \pm 7	(20) 27.5 \pm 2	(24) 23.0 \pm 1.5	(10) 9
Oral	(30) 255 \pm 21	(12) 275	(25) 225 \pm 27	(30) 250 \pm 16	(11) 210
Subcutaneous	(20) 270 \pm 60	(16) 260 \pm 21	(25) 195 \pm 33		
In rats					
Oral	(14) 280 \pm 50				
In guinea pigs					
Intraperitoneal	(20) 140 \pm 42			140 \pm 42	
In rabbits					
Intravenous	(14) 15 \pm 2.4			17 \pm 2	
Oral	(8) 600				
In dogs					
Intravenous	(14) 33				

* Figures in parentheses represent numbers of animals used

DISCUSSION. Although most of the known anti-histaminics are ethylenediamine derivatives or closely related compounds, it is apparent that anti-histamine activity is not limited to this structural type. Thephorin, a potent anti-histaminic, is entirely unrelated to existing compounds. It is remarkable that changes in the number of the double bonds cause such a striking decrease in activity. Little is known in general about the mechanism of action of anti-histaminics. It is very likely that Thephorin acts by competing with histamine for the receptive substance in the effector cell as it has been assumed for other anti-histaminics (17). The fact that the relative potencies of Thephorin, Benadryl and Pyribenzamine obtained from different tests are in good agreement lends support to the assumptions that the mechanism of action of these three substances is of similar nature. The linear relationship which exists between the amount of Thephorin and the dose of histamine to produce a constant pharmacologic effect favors the belief of a competitive mechanism. Similar results

have been obtained by Gaddum (18) for other drugs which supposedly compete for the same receptor. The antagonistic action of Thephorin against histamine is another example of "Specific Antagonism" according to Clark's (19) terminology. The remote possibilities of a chemical reaction with histamine or of accelerated histamine elimination remains, however, to be investigated.

The ineffectiveness of broncho-dilators, as epinephrine and aminophylline, against intracardially injected histamine in contrast to their protective action against inhaled histamine supports the view that their mechanism of action is different from that of specific histamine antagonists (9). The protection against histamine injected into the circulation seems to be the more reliable criterion for specific anti-histamine activity.

The greater efficacy of Thephorin and Benadryl in antagonizing histamine shock than anaphylactic shock in actively sensitized guinea pigs is in favor of the common belief that in addition to the release of histamine other as yet unknown factors may play an important role to bring about the symptoms of anaphylactic shock. An alternate interpretation has been suggested previously (11) and the discrepancy has been compared to the different action of atropine on the intestine against injected acetyl-choline and against vagal stimulation.

There is no apparent correlation between anti-histamine and local anesthetic activity since Nu-1525, which is practically devoid of anti-histamine activity "in vivo", is about as potent an anesthetic as Thephorin.

SUMMARY

Thephorin, 2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene, one of three closely related pyridindene derivatives studied, proved to be a potent anti-histaminic on isolated guinea pig's ileum, in the spray test, against intracardial histamine and against anaphylactic shock. In addition, Thephorin antagonizes spasmogenic effects of acetyl-choline, of Ba-ions and of epinephrine.

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THE QUANTITATIVE EVALUATION OF SPASMOLYTIC DRUGS IN VITRO¹

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For decades, the Magnus method (1) of recording the responses of excised strips of smooth muscle has been widely used for studying spasmogenic or spasmolytic activity. Fundamentally and technically, the method is simple and convenient. However, there is a striking lack of agreement in the techniques employed and in the manner of reporting and interpreting the responses so obtained. The desirability of uniform test procedures is obvious; possibly only slightly less evident is the need for improvement in handling the data. Of the reports on antispasmodics in the last decade, there is practically equal division between the authors who chose to draw conclusions on the basis of a complete abolition of spasm and those who attempted evaluation from partial spasmolytic effects. Two decades ago Trevan showed the error of expressing toxicity results and serum titers in terms of the minimum quantities required for 100% deaths or the maximum quantities tolerated without deaths. The same line of reasoning should be applied to the Magnus test method if quantitative interpretation of the results is to be attempted.

To meet this obvious need, an experimental design has been developed which is suitable for routine testing and which involves comparing each new drug with a suitable standard under reproducible conditions. This technic is generally applicable to the great variety of situations in which the Magnus method proves useful and indeed, represents an adaptation of the method described by Morrell, Allmark and Bachinski for the assay of the posterior pituitary hormone (2). An advantage of the procedure is that the influence of the minor variations in technic inevitably occurring even in the same laboratory on different days and with various experimenters is minimized if not eliminated. Further, the procedure yields definite potency estimates, the confidence limits of which can be determined by conventional statistical means. Such potency estimates facilitate comparisons of the relative activities of similar compounds, not only within the confines of a given laboratory but between laboratories.

As applied here to spasmolytic drugs, the procedure becomes an assay of a new compound in terms of an established reference drug such as atropine or papaverine. A choice of test responses is available since the drug under study may be allowed to antagonize the normal contractions of a surviving tissue strip, or to prevent the reaction to one of several spasmogenic drugs, or finally, to relax a state of tonus (spasm) previously induced by a spasmogen. The most useful

¹ Presented before the International Physiological Congress at Oxford, England in July, 1947.

² Deceased.

spasmogenic test drugs seem to be acetylcholine for parasympathetic and epinephrine for sympathetic actions, and the barium ion, oxytocic hormone, and histamine as musculotropic agents. The excised ileum of the rabbit is the best test object for barium or acetylcholine spasms whereas the guinea pig ileum responds best to histamine. For studies on the oxytocic hormone, the uterus of the guinea pig is preferred. However practically any smooth muscled organ may

TABLE 1

pD values equivalent to various dilutions and concentrations

pD	DILUTION RATIO	%	MG. %	MG./ML. (P.P.T.)	MG./L. (P.P.M.)
0.30	1:2	50	50,000	500	5 × 10 ⁵
0.60	1:4	25	25,000	250	2.5 × 10 ⁵
0.90	1:8	12.5	12,500	125	1.25 × 10 ⁵
1.00	1:10	10	10,000	100	1 × 10 ⁵
1.20	1:16	6.25	6,250	62.5	6.25 × 10 ⁴
1.50	1:32	3.12	3,120	31.2	3.12 × 10 ⁴
1.81	1:64	1.56	1,562	15.6	1.56 × 10 ⁴
2.00	1:100	1	1,000	10	1 × 10 ⁴
2.11	1:128	.781	781	7.81	7.81 × 10 ³
2.30	1:200	.5	500	5	5 × 10 ³
2.40	1:250	.4	400	4	4 × 10 ³
2.41	1:256	.39	39	3.9	3.9 × 10 ³
2.48	1:300	.333	333	3.33	3.33 × 10 ³
2.60	1:400	.25	250	2.5	2.5 × 10 ³
2.70	1:500	.2	200	2	2 × 10 ³
2.71	1:512	.195	195	1.95	1.95 × 10 ³
2.78	1:600	.167	167	1.67	1.67 × 10 ³
2.94	1:700	.143	143	1.43	1.43 × 10 ³
2.90	1:800	.125	125	1.25	1.25 × 10 ³
2.95	1:900	.111	111	1.11	1.11 × 10 ³
3.00	1:1000	.1	100	1	1 × 10 ³
3.01	1:1024	.0976	97.6	.976	9.76 × 10 ²
3.30	1:2000	.05	50.0	.5	5 × 10 ²
3.31	1:2048	.0488	48.8	.488	4.88 × 10 ²
4.00	1:10,000	.01	10.0	.1	1 × 10 ²
5.00	1:100,000	.001	1.0	.01	10
6.00	1:1,000,000	.0001	.1	.001	1
9.00	1:1,000,000,000	.0000001	.0001	.000001	.001

pD = Log of reciprocal of the dilution ratio. pD of pure compound is zero.

be employed provided appropriate test drugs are used. Papaverine hydrochloride was adopted as a standard of reference against musculotropic spasms, produced with either Ba⁺⁺ or histamine, and atropine sulfate against parasympathetic spasms induced by acetylcholine.

This experimental design did not obviate the difficulty of determining with suitable exactness the drug concentration just sufficient to produce the desired endpoint. That this endpoint is subject to normal biological variation is plainly evident in the abundance of literature reports giving the endpoint con-

centration as a range between two values differing by 100% or more. This inherent variability prompted the use of those statistical technics found applicable to such data in biological assaying which are based on the normal frequency distribution of responses to graded doses.

Finally there remained the problem of devising a convenient means of expressing the activity of antispasmodic drugs in absolute terms. The time-honored custom of reporting, as an expression of absolute potency, the strength of a drug in terms of a dilution is unsatisfactory in dealing with drugs differing widely in potency. Unwieldy numbers are unavoidable; they range from very small fractions to unity or from unity to very large dimensions depending on the point of entry into the metric scale. Therefore it has been found convenient to employ the concept used in expressing hydrogen ion concentrations as "pH" values by coining the term "pD".³ The pD is the "log dilution", or, expressed alternatively, the log of the reciprocal of the concentration. The simplification thus achieved in the numbers to be handled is illustrated in table 1, which presents pD values corresponding to a number of concentrations commonly used in pharmacology and bacteriology. Feinstone, et al, (3) have recently used "the logarithm of the reciprocal of the lowest tuberculostatic concentration" in a published graph. In this laboratory pD values have been used for two years to express the results of anti-bacterial tests.

Use of the term pD avoids the pitfall of applying the loose expression "dilution" when actually "concentration" is meant. Thus a 1:1000 "dilution" becomes simply pD 3.0 and a 1:200,000 "dilution" is pD 5.3. A further advantage is that the smaller 2 or 3 digit numbers are more easily grasped and remembered. Being based on the reciprocal of the corresponding concentrations, pD values increase with the activity of the drug and thus are a direct index to relative activity. Since they are continuous, pD values are capable of as exact expression as may be desired and are more convenient for graphic presentation.

PROCEDURE. A glass excised organ vessel with an internal aerating-mixing flue and a capacity of 150 ml. as shown in figure 1 was used. Two segments of muscle tissue were suspended simultaneously in this and were attached to individual writing levers. The vessel was hung in a waterbath at 37°C. and filled with pre-warmed Tyrode solution from a reservoir. The solution was aerated constantly with a slow stream of compressed air supplied through the internal flue. This method of aeration mixes added drug solutions much more quickly and thoroughly than conventional methods. By this arrangement, the responses of the two tissue segments to each application of test drug could be followed independently with one operator managing two such set-ups or 4 strips simultaneously. Such use of multiple strips is not new inasmuch as Morrell and co-workers (2) have described an assay for the posterior pituitary hormone in which eight strips of uterine tissue are suspended in a single bath.

As soon as the suspended strips had recovered from the contraction caused by the oper-

³ Since the presentation of this paper Schild (Brit. J. Pharmacol. 2, 189, 1947) has proposed the term pA which he defines "as the negative logarithm to base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose." It will be recognized that this is a restricted application of the concept herein presented.

active manipulation, the test was started. To produce a virtually maximum contracture, an excess of spasmogen was first applied to the resting strips; specifically, 0.15 mgm. of acetylcholine bromide or 15 mgm. BaCl_2 was used on rabbit ileum, or 0.03 mgm. of histamine hydrochloride for the guinea pig ileum. These doses were used routinely throughout the study as standard spasmogenic stimuli against which the spasmolytic powers of the standard and unknown were evaluated.

As soon as the full contracture from the spasmogen had developed, not over 0.5 ml. of a solution of the antispasmodic drug was added, without washing out the spasmogen. The degree of relaxation produced in the first two minutes was noted. In case there were any evidence that the maximum effects had not been developed in the two-minute interval, a longer period appropriate for the type of compound under study could be used. Following

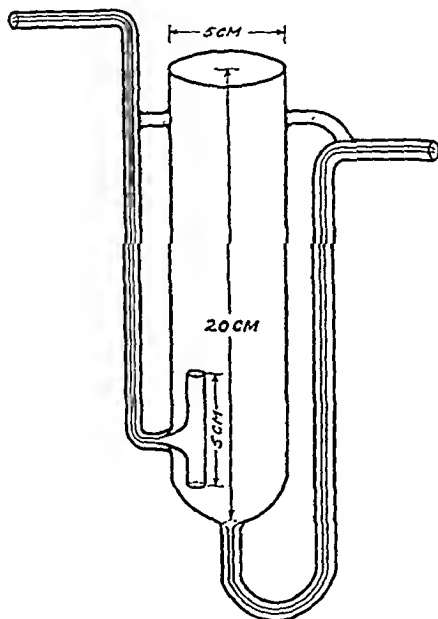


FIG. 1. VESSEL FOR EXCISED TISSUE BATH 150 ML. CAPACITY

the observation, the drug-containing Tyrode was flushed out and replaced with fresh solution. When the strips had relaxed to the former normal tonus level they were ready for another trial. Each pair of strips ordinarily was good for four or five trials unless excessive doses were applied or irreversible changes were encountered. It is preferable to keep constant the time interval between trials.

The data could be treated as graded responses by measuring the exact degree of relaxation produced by each dose and handling the values as described by Bachinski, Allmark, and Morrell (4). This is too tedious for routine work, and simpler rather than more complex methods were sought. This end was achieved by regarding the results as all-or-nothing in character. As suggested by Lands (5), a response was deemed "positive" if the level of contraction was returned

three-fourths of the distance to the control level during the two-minute observation period. When this was done, the data of table 2 were obtained for papaverine and atropine. It is obvious that these data are typically "all-or-none" and similar to dosage-mortality results of toxicity tests. They also plot as a straight line on coordinate graph paper when transformed into probits and pD values as shown in figure 2. Conventional statistical tests for linearity as well as for parallelism (6) show that a straight line adequately fits each set of points and that the lines for both atropine and papaverine are parallel within experimental limits.

Highly refined statistical treatment of the data is scarcely justified in view of the variable nature of the response, where at times the vagaries of random

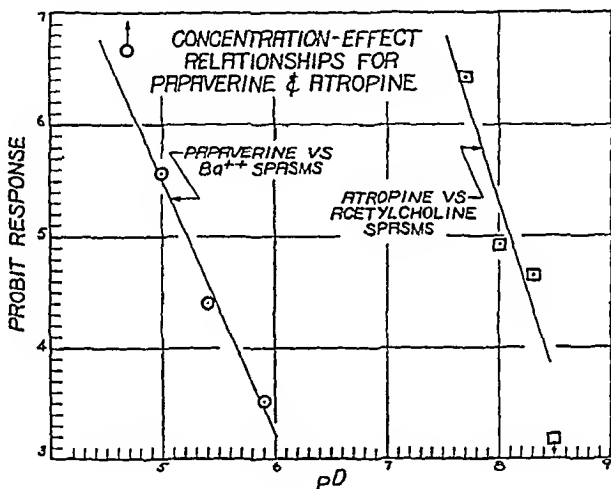


FIG. 2

"spike contractions" in the normal rhythm may determine whether or not the reduction in spasm produced by the drug amounts to a 75% reduction. The ED_{50} , that is, the drug concentration capable of producing positive responses in one-half of the strips tried, obtained graphically is sufficiently good for most purposes. Thus the probit of the response is customarily plotted against the concentration of the test drug and the best-fitting line is drawn by eye. The ED_{50} is read from the curve; being a measure of concentration in this case, it may be in conventional terms such as "per cent", "milligrams per cent", "mg./ml.", etc. or, as proposed herein, as a pD value. The approximate standard errors of these ED_{50} values may also be obtained graphically by the method of Miller and Tainter (7).

It should be emphasized that values obtained graphically are approximations. If the situation demands more precisely determined values and the data warrant the computational effort, the maximum likelihood solutions (8) may be cal-

culated. Both the computed and graphically estimated pD values of the ED_{50} and its standard error are given for the data in table 2; in this case they prove to be practically identical.

The potency of a new drug relative to an appropriate reference drug may be determined by either establishing a "standard" curve for the latter in a series of experiments or by testing the new drug and the reference compound in parallel. Though more time-consuming, the latter practice is the best means of avoiding

TABLE 2

Data showing the concentration-response relationships for the spasmolytic activities of papaverine against musculotropic spasms and of atropine against neurotropic spasms induced in excised rabbit ileum

PAPAVERINE (BARIUM-INDUCED SPASMS)			ATROPINE (ACETYLCHOLINE-INDUCED SPASMS)		
Drug concentration		Response: no. positive/no. tried	Drug concentration		Response: no. positive/no. tried
Mg %	pD		Mg. %	pD	
0.2	5.7	2/20	0.0003	8.5	0/8
0.4	5.4	6/22	0.0005	8.3	7/19
1.0	5.0	33/44	0.001	8.0	13/27
2.0	4.7	22/22	0.002	7.7	24/26
pD of ED_{50} (calculated)			5.23 \pm .05	8.09 \pm .05	
pD of ED_{50} (graphically estimated)			5.24 \pm .05	8.10 \pm .05	

TABLE 3

Dosage schedule for assay of two anti-spasmodic drugs against a standard on nine pairs of smooth-muscle segments

ORDER OF DOSING	SEGMENT PAIR NUMBER								
	1	2	3	4	5	6	7	8	9
1	a	z	b	y	b	x	a	z	c
2	x	a	z	c	y	b	x	c	z
3	b	y	a	z	c	y	b	x	a
4	y	b	x	a	z	c	y	b	x

Where letters a, b and c represent the low doses of the three drugs assigned at random, and x, y and z represent the high doses, also assigned at random.

errors introduced by variations in the sensitivity of the tissues and technic. A balanced design in which two concentrations each of the new drug and reference standard are applied to the strips in a predetermined order insures taking the data in such a way that the effects of variations in sensitivity between strips are cancelled out. Where four trials are made on each of four pairs of segments, a simple 4 x 4 design suffices for comparing a single compound against a standard. It is possible to compare two compounds against a standard by the design given in table 3. If six responses can be obtained on each of six pairs of segments, a 6 x 6 design may be applied.

The slopes of the concentration-effect curves in figure 2 are relatively flat indicating that ileac tissue is not capable of discriminating between very small increments in concentration of the antispasmodic. With papaverine acting against histamine on the guinea pig ileum, a three-fold increase in concentration is necessary to increase the proportion of positive responses from 1 in 5 (20%) to 4 in 5 (80%). Thus the accuracy of this assay is about the same as that of estrone on sprayed rats or the one-hour frog method (USP XI) for digitalis.

TABLE 4

Comparison of the antihistamine activities of Benadryl, Pyribenzamine and papaverine on the isolated guinea pig ileum

PAPAVERINE		BENADRYL		PYRIBENZAMINE	
pD	Result	pD	Result	pD	Result
5.3	2/12	7.3	2/12	7.6	5/12
5.0	7/12	7.0	11/12	7.3	10/12
pD of ED 50	5.06	7.20		7.54	
Potency. . .	1	126 \pm 23		305 \pm 84	

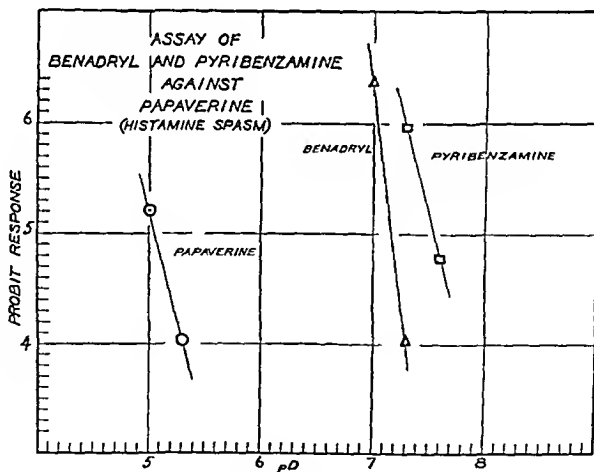


FIG. 3

RESULTS. An illustration of the application of this method is the comparison of the two new antihistamine drugs, Benadryl and Pyribenzamine, with papaverine in reducing histamine-induced spasms in guinea pig ileac muscle. The experiment was set up so that the effectiveness of two concentrations of each of the three drugs were compared in a sequence determined by a balanced design pattern similar to table 3, against the spasm induced by 0.03 mgm. of histamine. The results of the comparison are shown in table 4 and are portrayed graphically in figure 3.

These data show that Benadryl and Pyribenzamine are much more effective in relaxing histamine-induced contractions in guinea pig ileum than is papaverine. The calculated (7) ratios are 126:1 and 305:1 for Benadryl and Pyribenzamine, respectively. Because only 12 strips were used in this assay on each of the two concentration levels and the guinea pig ileum is less discriminatory than might be desired, the average standard errors associated with these values are apparently large, namely, ± 23 and 84 or 18% and 28% of the respective ratios. However, these errors are small compared to those encountered in most pharmacodynamic measurements on Magnus preparations, and the difference between the activities of the two drugs is well beyond the bounds of normal chance variation ($P < .01$). By replication, the accuracy of the estimate of the activity can be easily pushed to any degree needed for practical interpretation of the results. To test the reproducibility of the method, the experiment which yielded the data given in table 4 was repeated twice about three months later. The individual ED_{50} values were 5.11 and 5.12 for papaverine, 7.28 and 7.44 for Benadryl and 7.65 and 7.63 for Pyribenzamine. The calculated potencies indicated by the combined data of these two assays were 185 ± 56 and 336 ± 55 for Benadryl and Pyribenzamine, respectively. Since these results did not differ significantly from those in table 4 they were combined into weighted averages; these were 140 ± 22 for Benadryl and 328 ± 46 for Pyribenzamine. Comparison of the results of these three sets of experiments shows the values obtained in any one set were reliable to limits well within that required for the interpretation of significant differences for this type of experimentation.

Sherrod, Loew and Schloemer (9) recently published data comparing Benadryl and Pyribenzamine which indicate that the latter is somewhat more active than Benadryl in preventing the fall in blood pressure from histamine injections in the anesthetized dog. Pyribenzamine was also shown by the same authors to have greater anti-asthmatic potency in guinea pigs subjected to breathing histamine aerosols. Thus the data obtained *in vitro* by the test procedure here proposed agree qualitatively with those seen in the intact animals.

SUMMARY

An experimental procedure is described for using the Magnus-type excised organ preparation for comparison of the spasmolytic potency of compounds under test against standardized spasms induced by fixed doses of barium, histamine or acetylcholine. The proportion of preparations giving a positive response at different dose levels is changed to straight line functions by using probit and log-dose transformations. From these the ED_{50} and ratios of activity can be readily determined by conventional methods.

Use of the symbol pD is proposed to express the concentrations required for a standard effect. This value is the logarithm of the reciprocal of the concentration of the active drug or, expressed alternatively, the log of the degree of dilution. It thus is entirely comparable to the term pH , now widely accepted as a convenient means of expressing otherwise unwieldy numerical values of hydrogen ion concentration.

This general assay procedure may be readily adapted to quantitating the responses of any smooth muscled organ to tonic or depressant drugs by appropriate choice of the standard and test agents.

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OBSERVATIONS ON THE COMPARATIVE PHARMACOLOGIC ACTIONS OF 6-DIMETHYLAMINO-4,4-DIPHENYL-3-HEPTANONE (AMIDONE) AND MORPHINE¹

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The attempt to find new analgesic compounds preferable to morphine or other opium alkaloids met with little success until 1939 when Eisleb and Schaumann (1, 2) reported the action of isonipecaine (dolantin, demerol), a synthetic compound possessing both morphine-like and atropine-like activity. Recently, an investigating team headed by Dr. E. C. Kleiderer has made available information on related compounds prepared by German chemists in a report published by the U. S. Department of Commerce (3). The most promising agent of the many described appears to be 6-dimethylamino-4,4-diphenyl-3-heptanone, which bore the German serial number 10820, and which has been referred to since its introduction into this country also as amidone and dolophine. In the following account the compound will be referred to by the name amidone.

EXPERIMENTAL METHODS AND RESULTS. 1. *Comparative Acute Toxicities to Rats.* Male albino rats were used, ranging in weight from 100-175 gm. Food was withheld for 4-6 hours prior to administration of the test materials. Acute oral, subcutaneous and intravenous toxicity studies were made. A 4% morphine sulfate solution was used in all tests involving this material. Similarly a 4% solution of amidone hydrochloride was used except for the intravenous toxicity studies for which a 0.4% solution was employed.

At least 4 groups of 10 rats were used for each route of administration for each drug. The results are summarized in Table I. LD₅₀'s were determined by calculating the regression line for the data in log dose-probit units (all-or-none data were excluded from the calculation). The standard errors were also evaluated by adapting log dose-probit units to a formula described by Snedecor (4).

The results show that in the rat amidone is, on a weight-for-weight basis, about 10 times more toxic than morphine orally, about 6 times more toxic subcutaneously, and about 25 times more toxic intravenously. As judged by the standard errors it appears that oral and subcutaneous absorption of amidone is more irregular than that of morphine.

2. *Subacute Toxicity Studies.* a) *Comparative subacute toxicities to rats:* Albino rats were used. Finely ground Purina Dog Chow served as the basic diet, and into this was incorporated with thorough mixing the materials to be studied. Dietary levels of amidone hydrochloride and morphine sulfate used were: 0.01, 0.05, 0.1, 0.25, 0.5 and 1.0%. Groups of 12 rats, equally divided as to sex, were fed, ad libitum, each of the above dietary levels

¹ These investigations were supported by a grant from Bristol Laboratories, Inc., Syracuse 1, New York.

TABLE I
Acute toxicity of amidone and morphine to rats

ROUTE OF ADMINISTRATION	DOSE	MORTALITY RATIO	AV. TIME OF DEATH	LD ₅₀ ± S.E.
Amidone Hydrochloride				
Oral	mg./kg. 120	6/10	hr. ca 8	mg./kg. 95 ± 33
	100	6/10	ca 3	
	90	3/10	ca 2	
	80	6/10	ca 2	
	70	3/10	ca 7	
Subcutaneous	120	7/10	ca 3	100 ± 19
	100	5/10	ca 7	
	90	3/10	ca 6	
	80	4/10	ca 5	
	70	2/10	ca 2	
	60	3/10	ca 2	
	40	0/5	—	
Intravenous	20	5/5	.1	9.2 ± 0.4
	15	5/5	.2	
	10	7/10	.17	
	9	4/10	.17	
	9	4/10	.17	
	8	3/10	.17	
Morphine Sulfate				
Oral	1000	7/10	ca 4	905 ± 144
	900	3/10	ca 6	
	800	5/10	ca 3	
	700	2/10	ca 3	
Subcutaneous	600	7/10	ca 7	572 ± 6
	575	5/10	ca 6	
	575	6/10	ca 6	
	550	3/10	ca 7	
	400	0/5	—	
Intravenous	300	5/5	.07	237 ± 6
	250	8/10	.07	
	240	5/10	.18	
	230	5/10	.07	
	220	1/10	4	
	200	0/5	—	

of these materials, and in addition 6 of each sex served as controls and received the unadulterated diet. The duration of the experiment was 100 days, and the weight of each rat was recorded once a week during this period.

The results (see Table II) showed that, in general, an increasing depression in growth accompanies increasing dietary concentrations of both amidone and morphine and that the degree of this is of similar order for both materials except at the higher concentrations at which amidone produced the greater weight depression. So far as could be judged, both types of diet were accepted equally well.

Increased mortality was apparent for amidone at 1% dietary concentration for males and at 0.5% for females. The corresponding dietary concentrations

TABLE II
Effect of various dietary levels of amidone hydrochloride and morphine sulfate on growth and survival of rats over a 100 day period

CONC. IN DIET	AMIDONE			MORPHINE		
	Average Body Weight		Mortality Ratio	Average Body Weight		Mortality ratio
	Original	Change at 100 days		Original	Change at 100 days	
Male						
%	gm.	%		gm.	%	
none	123	+186	1/6	123	+186	1/6
0.01	123	+172	2/6	121	+175	1/6
0.05	121	+140	0/6	121	+141	1/6
0.1	121	+148	0/6	121	+169	1/6
0.25	121	+113	0/6	123	+117	0/6
0.5	123	+49	2/6	123	+139	2/6
1.0	123	+15	5/6	123	+60	5/6
Female						
none	115	+110	1/6	115	+110	1/6
0.01	117	+103	0/6	117	+87	1/6
0.05	113	+80	1/6	116	+62	1/6
0.1	114	+76	0/6	116	+81	0/6
0.25	117	+53	1/6	117	+46	3/6
0.5	115	+90	5/6	115	+73	1/6
1.0	115	—*	6/6	114	+51	2/6

* None survived more than 2 weeks and all lost weight.

for morphine were 1% for males and seemingly greater than 1% for females.

Histopathologic examinations were made on animals selected from the groups receiving the higher dietary levels of amidone and morphine and also upon 4 control animals. The resultant findings are summarized in Table III.

The only pathologic findings which could be interpreted as related to the action of amidone or morphine were the frequently occurring pneumonias, sometimes associated with patchy atelectasis. The respiratory depression produced by these drugs may have played a role in this. Hemosiderosis of the spleen as seen in two animals is seen too often in rats under normal conditions

to be considered of significance. The slight parenchymatous changes of the liver seen in 3 animals was associated in 2 with massive pulmonary infection.

TABLE III

Histopathologic findings in certain of the rats receiving amidone and morphine in their diets

DRUG	DIETARY CONC. OF DRUG	SEX	TIME OF DEATH	HISTOPATHOLOGIC FINDINGS
	%		days	
Control	0	M	100*	Negative
	0	M	100*	Negative
	0	F	100*	Negative
	0	F	100*	Negative
Amidone	1	M	11	Grayish nodules in lung, patchy atelectasis and pneumonia. Slight hemosiderosis of spleen
	1	M	100*	Mild hemosiderosis of spleen
	1	F	9	Patchy atelectasis of lungs
	1	F	12	Necrotizing multiple pneumonia
				Cloudy swelling of liver cells
	0.5	M	100*	Slight cloudy swelling of liver cells
	0.5	M	52	Encapsulated liver abscesses and suppurative lymphadenitis (coccidiosis). Patchy atelectasis and hyperemia in lungs
	0.5	F	20	Multiple purulent pneumonic foci in lungs
	0.5	F	100*	Hyperplasia of splenic pulp Negative
Morphine	1	M	93	Hydropic swelling of liver cells
	1	M	10	Multiple purulent pneumonic foci
	1	F	100*	Negative
	0.5	M	12	Patchy pneumonia. Serous exudation in lungs
	0.5	M	100*	Negative
	0.5	F	100*	Negative
	0.5	F	100*	Negative

* Killed upon termination of the experiment.

The liver infestation seen in 1 of the animals receiving amidone was undoubtedly coincidental to the treatment it received.

b) *Comparative subacute toxicities to dogs*: Eight female mongrel dogs were used for this study and were divided into 4 groups of 2 each. One pair received a subcutaneous injection of amidone hydrochloride at a dose of 2 mg. per kg. daily excepting Sundays. The second pair received amidone hydrochloride subcutaneously on a similar schedule in a dose of 5 mg. per kg. The other 2 pairs of dogs received 2 and 5 mg. doses of morphine sulfate on the same schedule. Both drugs were used as a 4% aqueous solution.

Approximately 2 weeks before the experiment began, episiotomy was done on each animal to facilitate urine collection. Just prior to the beginning of the experimental period,

the following observations were made: bromsulfalein retention, phenolsulfonphthalein excretion, blood non-protein nitrogen, red cell count, and hemoglobin (acid hematin) values. Blood smears were also made at this time. At the time of administration of the first dose of the drug, the blood sugar response (venous) was determined, using the micro-method of Folin and Malmros (5). At 50 and 100 days after the beginning of the experiment, all the above observations were repeated on each animal. The experiment was terminated and the surviving animals sacrificed at 100 days.

It will be noted from Table IV that most of the animals, particularly the 2 that died during the experiment, displayed marked weight losses throughout the course of observation. That this may be due to the hypnotic and analgesic

TABLE IV

Body weight changes in dogs receiving daily subcutaneous doses of amidone hydrochloride and morphine sulfate

DRUG AND DOSAGE	ANIMAL NUMBER	AVERAGE BODY WEIGHT		
		Original	Change at:	
			50 days	100 days
		kgm.	%	%
Amidone 2 mgm./kgm./day	1	18.5	-27.0	-29.7
	2	6.4	+17.2	+20.3
	3	15.5	-26.5	-25.8
	4	9.3	-40.9	died
Morphine 2 mgm./kgm./day	5	14.9	-14.1	-3.4
	6	15.2	-21.7	-19.7
	7	9.4	-35.1	died
	8	8.9	-13.5	-15.7

effects of the agents, and consequent reduction of food intake, is suggested by the partial reversal of this weight loss tendency in the animals receiving the lower dose of morphine, to the effects of which relative habituation ultimately occurred.

None of the functional tests (kidney, liver) showed abnormalities after the 100 day administration of either of the drugs. NPN values were likewise essentially unchanged. Red cell counts and hemoglobin concentrations decreased slightly in all of the dogs receiving amidone excepting 1 on the 2 mg. dose level in which there was no change. Of those receiving morphine, red cell counts and hemoglobin concentrations were unchanged at the 2 mg. dose level, markedly decreased at the 5 mg. level in the dog that died, and slightly decreased in the other one. Blood smears taken from the dogs receiving amidone showed evidences of slight hypochromic microcytic anemia in both of the animals at the higher dosage and in 1 of those receiving the lower dose of amidone. This

change was seen only at the higher dosage in those animals receiving morphine, being marked in the 1 dog that died.

Table V shows the effects of amidone and morphine on the blood sugar level and the change in this response upon repeated administration of the drugs. It will be noted that tolerance to the blood sugar response occurred earlier (in all but 1 animal) to morphine than to amidone.

Histopathologic examination was done on all 8 of the dogs. The resultant findings are summarized in Table VI. The occurrence of massive hemorrhages (spleen, lung, stomach) could not be explained on the basis of changes in the vascular wall since no anatomical lesions were found. Although studies on blood clotting mechanisms were not done, these hemorrhages might have been related to an alteration in coagulability suggestive of which are the findings of Pugliese (6) and Doyon (7) in regard to the effect of intravenously injected morphine on the coagulability of dog's blood.

DISCUSSION. The results of our LD₅₀ determinations on rats show that the acutely lethal dose for amidone is markedly smaller than that for morphine, the smallest difference found (subcutaneous) being of the order of 6 fold. From data on intravenous toxicity to mice (8, 9), rats seem to be about twice as susceptible to amidone by this mode of administration. Acute toxicity data for other species are not available.

In contrast to the findings in the acute experiments, the subacute toxicity studies indicate that on a chronic basis the toxicity of amidone to rats compares much more closely with that of morphine. Although Scott and Chen (8) made no comparison with morphine the results of our subacute toxicity studies on amidone agree, in general, with theirs. We have made no acute toxicity studies on dogs, but our subacute toxicity data for this species indicates amidone to be only slightly more toxic than morphine, and then only on the basis of changes in the blood picture.

From a visual standpoint, the most striking difference between these drugs appeared in the dog experiments in connection with degree of tolerance developed to their depressant effects. Whereas, initially both agents produced approximately equal depth of depression, during the course of the experiment this response to morphine became progressively weaker. In contrast, with amidone, depth of depression remained relatively unchanged although there seemed to be a tendency for it to become of somewhat shorter duration. This relative lack of development of tolerance to the hypnotic action of amidone has previously been noted (10), although it is now apparent that tolerance may develop to certain of its other actions; analgesia (11, 12), sedation and hypothermia (12), hypotensive action (13).

Another action of amidone to which tolerance develops has been brought out in our blood sugar studies. The only other study on the blood sugar response to amidone that has come to our attention is that of Isbell and coworkers (14) who found no rise in blood sugar in man even with doses up to 75 mg. Hence a species variation appears to exist as regards blood sugar response to amidone.

TABLE V

Blood sugar response to amidone or morphine in dogs receiving chronic subcutaneous doses

DOG NUM- BER	TREATMENT	BLOOD SUGAR RESPONSE															
		First day					50 days					100 days					
		Control level	Change (mgm.%) at:				Control level	Change (mgm.%) at:				Control level	Change (mgm.%) at:				
			1 hr.	2 hr.	4 hr.	6 hr.		1 hr.	2 hr.	4 hr.	6 hr.		1 hr.	2 hr.	4 hr.	6 hr.	
		mg. %					mg. %						mg. %				
1	Amidone hydrochloride—2 mgm./kgm./day subcuta- neously	75	+56	+72	+44	+32	92	+37	+7	+f	+4	92	+1	0	-7	-1	
2		96	+5	+67	+27	+1	88	+11	+5	+1	+0	90	-4	-1	-6	-9	
3	Amidone hydrochloride—5 mgm./kgm./day subcuta- neously	92	+51	+54	+31	+11	95	+90	+60	-1	-29	89	-4	-2	-18	-18	
4		83	+26	+22	+54	+37	140	+154	+89	-29	-78		Died at 72 days				
5	Morphine sulfate—2 mgm./ kgm./day subcutaneously	89	+65	+52	+21	-1	101	+3	-1	-12	-12	89	-5	-2	-11	-12	
6		109	+102	+48	+1	-3	105	-4	-5	-5	-11	96	-8	-11	-11	-12	
7	Morphine sulfate—5 mgm./ kgm./day subcutaneously	90	+98	+71	+32	+15	89	+3	+12	-6	-8		Died at 70 days				
8		93	+74	+92	+21	+10	91	+105	+51	-1	-9	94	-8	+13	+8	-6	

TABLE VI

Histopathologic findings in dogs receiving daily subcutaneous doses of amidone or morphine

DOSAGE	ANIMAL NUMBER	HISTOPATHOLOGIC FINDINGS
Amidone		
mgm./kgm. 2	1	Hydropic swelling of liver cells. Slight hemosiderosis of spleen.
	2	Hydropic swelling of liver cells. Cystitis.
5	3	Angiomatous nodules in lung.
	4	Massive gastric hemorrhage. No gross lesions or definite microscopic changes in gastric mucosa.
Morphine		
2	5	Multiple subcapsular hemorrhages in spleen unaccompanied by vascular or other changes.
	6	Massive unilateral hemorrhage in lung. Bronchi filled with blood. No vascular changes or thromboses.
5	7	Massive heart-worm infestation. Moderate fatty changes in liver and renal tubular epithelium. Moderate hemosiderosis of spleen.
	8	Negative.

SUMMARY

By acute oral, subcutaneous and intravenous administration to rats, amidone is several times more toxic than morphine. On a subacute basis, amidone is but slightly more toxic than morphine to rats and dogs.

There is little evidence of development of tolerance to the depressant action of amidone in dogs.

Amidone produces a hyperglycemic response in dogs to which tolerance develops on continued administration.

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THE EFFECT OF ETHER AND DIAL-URETHANE ANESTHESIA ON THE PASSAGE OF SULFATHIAZOLE INTO THE CEREBROSPINAL FLUID¹

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The factors which control the permeability of the barrier between the blood and the cerebrospinal fluid (csf) have not yet been elucidated. Examples of a change in barrier permeability are given in reviews by Katzenelbogen (1), Flexner (2), and Greenfield (3). It is generally recognized that the barrier to certain drugs or dyes may be lowered during high fever, in acute inflammation of the meninges, and after traumatic injury to the central nervous system. Although there have been studies, largely by Stern, which indicate that certain drugs might modify the permeability of the barrier with respect to another substance (1), there has been no systematic attempt to analyze the factors concerned.

The older literature on the production of csf considered the problem of its elaboration by the chroid plexuses (4). The recent studies of Wallace and Brodie (5) on the passage of bromide and iodide ions into the csf indicate that the extracellular fluid of the central nervous system is of greater importance as a source of csf than previously recognized. It follows from these studies that attention should be directed towards the meningeal and intracerebral blood vessels as well as the choroidal vessels in considering factors concerned with the production of csf and the passage of substances from the blood into the csf. The blood-csf barrier thus becomes accessible to experimental investigation from the standpoint of cerebral circulatory changes.

Accordingly, the experiments reported here were undertaken in order to determine whether ether anesthesia which produces cerebral vasodilatation (6, 7, 8) and increases brain blood flow (9) is accompanied by an increase in the penetration of a foreign substance into the csf. The findings are compared with dial-urethane anesthesia, neither of which drugs alone dilates cerebral vessels (9, 11, 12). We chose to follow the passage of sulfathiazole into the csf because of the ease of its chemical determination and because of its possible value in the treatment of certain meningitides.

METHODS. Two types of experiments were performed on fasting dogs: one in which sulfathiazole was injected into the peritoneal cavity, the other in which it was injected into the duodenum. We preferred the intraduodenal route because the blood level thus produced did not rise so high nor fall so quickly as after intraperitoneal injection, but as this

¹ Aided by a grant from the University Research Committee. Preliminary abstracts, have been published (*Federation Proc.*, 4: 132, 1945; 5: 207, 1946; *J. Alabama Acad. Sci.*, 18: 75, 1946).

required anesthesia the latter method was also employed in order to obtain control values in unanesthetized animals. In the experiments utilizing the intraduodenal route the dogs were anesthetized with ether or with dial-urethane solution² (0.6 cc./kgm. intraperitoneally) and a femoral vein blood sample and a 0.6 cc. sample of csf by cisternal puncture were taken for blank determinations. A tracheal cannula was inserted when ether anesthesia was employed. The abdomen was opened, the pylorus ligated, and 10 cc./kgm. of a 2.4 per cent sulfathiazole sodium sesquihydrate solution introduced into the duodenum below the ligature. Blood and csf samples were taken at hourly intervals up to five hours after sulfathiazole administration. The temperature of the dogs was maintained with an electric lamp when necessary.

In experiments in which sulfathiazole was given intraperitoneally the dose was varied and dial-urethane solution when used was given intravenously (0.5 cc./kgm.). Blood and csf samples were taken for analysis three hours after sulfathiazole injection. A thirty minute blood level was also determined to insure an adequate rate of absorption. Results on dogs whose blood level at thirty minutes was less than that at three hours were discarded. Free sulfathiazole levels were determined in duplicate by the method of Lee, Hannay and Hand (12), using a Coleman Model 11 spectrophotometer or a Cenco Photometer.

RESULTS. *Intraduodenal administration of sulfathiazole.* The average blood and csf sulfathiazole levels after intraduodenal injection in both ether and dial-urethane anesthesia are shown in fig. 1. In giving the same dose of sulfathiazole to all animals in this series, it was our intention to produce the same blood sulfathiazole levels in both groups of animals. It was an unexpected finding that the blood sulfathiazole levels during ether anesthesia were higher than those with dial-urethane.³

For our present purposes it is more pertinent that the csf sulfathiazole levels under ether anesthesia were higher than under dial-urethane (fig. 1). In order to compare the relative csf levels, C/B ratios in each experiment were calculated. These C/B ratios were obtained by dividing the three hour csf level by the three hour blood level. The data are shown in Table I. These findings indicate that *there is an increased passage of sulfathiazole into the csf in ether anesthesia.* Levels at the third hour were selected in calculating the C/B ratios because (1) about three hours were required for the csf values to reach a plateau under the conditions of the experiments, both after intraduodenal and intraperitoneal injection and (2) the C/B ratios at this hour have a smaller standard deviation than those calculated from blood levels at any other time.

Intraperitoneal administration of sulfathiazole. In this series of experiments data on unanesthetized as well as anesthetized animals were obtained. Table

² Supplied through the courtesy of Dr. F. L. Mohr of Ciba Pharmaceutical Products Inc. "Dial with Urethane Solution" is said to contain in each cubic centimeter 0.1 gram of diallylbarbituric acid, 0.4 grams of urethane and of monoethylurea.

³ The difference between the mean blood levels at the second, third, and fourth hour were significant ($P < .01, .02, .02$ respectively by Student's t test), though not at the first and fifth hour. A similar result was found in animals receiving sulfathiazole intraperitoneally. The third hour blood level per gram dose was significantly higher under ether anesthesia than in unanesthetized animals. That under dial was higher than normal but not significantly. We suggest that the higher blood levels may be due to one of the following effects of ether on sulfathiazole, alone or in combination: an increased rate of absorption, a decreased rate of conjugation, merely an apparent increase in blood level due to hemoconcentration. None of these possibilities have been investigated.

It shows the mean C/B ratios obtained at the third hour after sulfathiazole injection. A comparison of the C/B ratios confirms the previous findings in that there is a greater transfer of sulfathiazole to the csf during ether anesthesia than

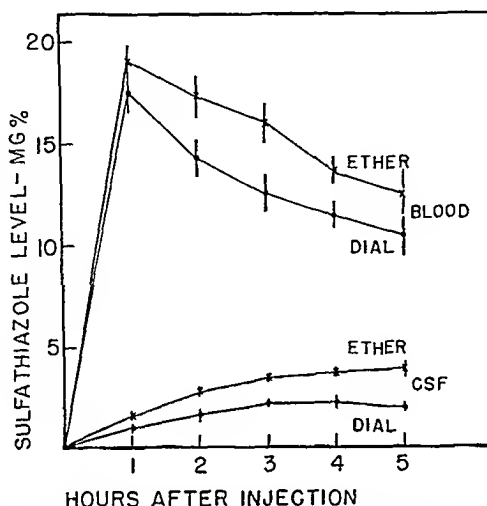


FIG. 1. Average blood and csf sulfathiazole levels following intraduodenal administration of 0.24 gram/kgm. of sulfathiazole sodium sesquihydrate to dogs anesthetized with ether or with dial-urethane. Vertical bars indicate one Standard Error of the Mean for small samples $\left(= \sqrt{\frac{\sum d^2}{N(N-1)}} \right)$ Average of thirteen experiments with each anesthetic.

TABLE I

Average C/B ratios three hours after injection of sulfathiazole sodium sesquihydrate in dogs

NUMBER OF EXPERIMENTS	ANESTHETIC	SULFATHIAZOLE INJECTION ROUTE	MEAN C/B RATIO \pm S.E.*	% CHANGE IN MEAN C/B RATIO	††	SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS
13	Dial-urethane i.p.	Duodenum	0.176 \pm .0083			
13	Ether	Duodenum	0.227 \pm .0089	+29.0	4.219	P < .01
15	No anesthesia	I.P.	0.138 \pm .0031			
12	Dial-urethane i.v.	I.P.	0.165 \pm .0038	+19.6	5.489	P < .01
10	Ether	I.P.	0.248 \pm .0090	+79.7	13.336	P < .01

* Standard error of the mean for small samples.

† Obtained by Student's *t* test.

during dial-urethane anesthesia. It also shows that there is less passage of the drug in unanesthetized animals than with either type of anesthesia.

Conclusions drawn from the C/B ratios as shown above are based upon the assumption that there is a straight line relationship between the blood and csf

levels of sulfathiazole. As fig. 2 indicates there is a high direct linear correlation between the csf and blood levels, for as the blood levels rise there is no disproportionate increase in the csf values, at least within the range studied. The three regression lines in this figure indicate graphically the differences between the C/B ratios for the three groups of animals.

DISCUSSION. The point of departure in initiating these experiments was the conception gained from the literature that ether dilates cerebral vessels and dial-urethane does not. The evidence for this is as follows: In 1930 Fulton, Liddell and Rioch (10) observed that the meninges and cortices of dogs, cats and monkeys were relatively bloodless under dial anesthesia, contrasting markedly with the vascular engorgement commonly seen under ether. Forbes, Finley and Nason

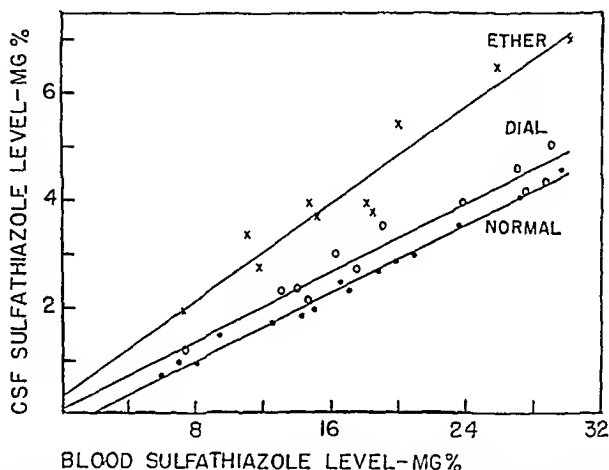


FIG. 2. Correlation of blood and csf sulfathiazole levels three hours after intraperitoneal injection of sulfathiazole. The regression lines were fitted by the method of least squares. Their coefficients of correlation, corrected for small samples, are as follows: unanesthetized group, $\bar{r} = +0.993$; dial group, $\bar{r} = +0.975$; ether group, $\bar{r} = +0.958$. The equations for each line are respectively, $Y = -0.310 + 0.160X$, $Y = 0.0724 + 0.161X$, $Y = 0.331 + 0.226X$.

(11) found little pial vasodilatation in cats and monkeys under dial. Other barbiturates have been reported to dilate pial vessels (6, 7), but not to the extent produced by ether (6, 7, 9). Urethane was used as an anesthetic by Schmidt and Hendrix (9) to obtain basal readings before producing cerebral vascular changes. We feel that it is fair to assume at least that the caliber of the cerebral vessels in the dog is larger under ether anesthesia than under dial-urethane.

Further studies, to be reported later, have made it clear that the caliber of the cerebral vessels is not the only important factor in determining the passage of sulfathiazole into the csf. For example chloroform anesthesia, like ether, dilates cerebral vessels (7) but was not found to be accompanied by an increased sulfathiazole concentration in the csf.

Thus the factors which bring about higher csf sulfathiazole levels in ether anesthesia are undoubtedly more complex than simply the production of cerebral vasodilatation. In searching for these factors, one must consider the fact that anesthesia with ether differs markedly from that with dial-urethane. For example, variations in oxygen and carbon dioxide content of the blood probably occur more frequently during ether anesthesia, because of the difficulty of maintaining dogs in light surgical anesthesia for three hours or more.

Oxygen saturation and carbon dioxide levels were determined on femoral arterial blood in some animals three hours after ether and dial-urethane anesthesia had been in progress (intraperitoneal series). Under ether anesthesia, oxygen saturation was found to be higher and carbon dioxide content lower than under dial-urethane. Although these findings were not surprising, they do not explain the cerebral vasodilatation that occurs with ether, since it is recognized that cerebral vasodilatation is favored by anoxia and by hypercapnia (13).

It is probable that the arterial blood pressure is also an important factor. Blood pressure is generally considered to be normal in both ether and barbiturate anesthesia (14). We recorded the femoral arterial pressure in a few dogs and after three hours found it to be usually somewhat lower with ether anesthesia than with dial-urethane.

A logical explanation of the changes in csf concentration of a substance from the standpoint of hemodynamics would include both the factors of arterial pressure and the diameter of the cerebral vessels. The resultant of these factors is the total brain blood flow. If to this is added a consideration of the state of the cerebral capillaries, we believe that it might be possible to account for the differences in csf levels. Although little can be said at present about the control of cerebral capillary permeability, a great deal of information is available as to the total brain blood flow largely from the work of Schmidt (13), who found that total brain blood flow in the cat, monkey and rabbit was increased by ether as compared to pentobarbital or urethane. Unfortunately for our present studies, the dog was reported to be a poor subject for total brain flow determinations and dial-urethane was not the anesthetic used. If we can assume that ether probably increases total brain blood flow in the dog as compared with dial-urethane, then we believe that our findings can be explained on a basis of hemodynamics.

We would like, therefore, to offer the tentative hypothesis that *variations in passage of any given substance from the blood into the csf are regulated (1) by the total brain blood flow and (2) by the state of the cerebral capillaries*. It is to be understood that we are referring to that moiety of a substance in the blood not bound to plasma proteins.⁴ We recognize that some substances, for reasons not

⁴ We have not lost sight of the fact that a large part of the blood sulfathiazole is bound to blood protein and that this accounts for the observations of others that csf sulfathiazole levels are lower than those of some other sulfonamides (15). This, however, does not affect our argument since Davis (15) has shown that the amount bound is inversely proportional to the amount present in blood and constant for any given level. We did not attempt to determine the percentage bound by protein. If this is taken into consideration, it would change the slope of the regression lines and the absolute C/B ratios but it would not alter our conclusions.

yet obvious, pass the barrier in lower concentrations than others. Further discussion of this hypothesis would not seem to be profitable at this time. It should be noted, however, that clinical experiences have shown that higher csf sulfathiazole values are found in some cases of meningitis than normally (16).

SUMMARY

The passage of sulfathiazole from the blood to the csf was compared in dogs under ether and under dial-urethane anesthesia after intraduodenal and after intraperitoneal administration of sulfathiazole. Unanesthetized dogs were also studied after intraperitoneal injection. The transfer of sulfathiazole was indicated by the C/B ratios obtained by dividing the csf level by the blood level at the third hour after administration of the drug.

It was found that there was an increased level of sulfathiazole in the csf during ether anesthesia. After intraduodenal injection, the C/B ratios were 29% higher with ether anesthesia than dial-urethane. After intraperitoneal injection the C/B ratios under dial-urethane were 20% higher than in unanesthetized animals and under ether they were 80% higher. It was also noted that higher blood sulfathiazole levels were obtained under ether anesthesia.

These findings were discussed from the standpoint of the influence of changes in cerebral circulation. It is thought that the transfer of sulfathiazole to the csf may be regulated by the total brain blood flow.

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THE PHARMACOLOGY OF COMPOUNDS RELATED TO β -2,5-DIMETHOXY PHENETHYL AMINE

I. THE ETHYL, ISOPROPYL AND PROPYL DERIVATIVES

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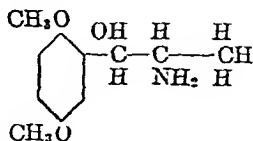
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INTRODUCTION. The following paper presents the results of a systematic study of a series of phenethylamine derivatives. The individual members were examined for toxicity, circulatory effects, mydriatic action and action on the isolated intestine and uterus. The data obtained were analyzed with respect to (a) absolute potencies, (b) the relationship of pharmacological action to chemical structure, and (c) correlations between various pharmacological effects and pressor activity, which is the outstanding, although not the common, feature of the group.

Discussion will be confined solely to the data presented in this paper since it frequently happens that relationships between chemical structure and physiological action are limited to a given series. Reviews such as that by Hartung (1) may be consulted for the broader aspects of this problem.

The conclusions drawn are dependent upon the precision and accuracy of the data in question; therefore, every effort was made to reduce error by careful experimentation and by the use of as many experimental animals as was practicable. All physiological measurements were standardized and each was carried out by the same operator insofar as possible. All 24 compounds were synthesized and analyzed in these laboratories (2, 3, 4).

The plan of the series is as follows: the 24 members possess as a common feature an amino or quaternary nitrogen linked through a two-carbon chain to a 2,5-dimethoxyphenyl group so that the latter is in a beta-position, with respect to the nitrogen group. The 18 amine hydrochlorides consist of the primary, secondary and tertiary derivatives of the following 6 alkyl side chains: ethyl, isopropyl, propyl, β -hydroxyethyl, β -hydroxyisopropyl and β -hydroxypropyl. For purposes of discussion it is sometimes convenient to regard the propyl derivatives as β -methylethyl compounds. In addition, the quaternary ammonium chlorides, which are different chemically and often physiologically from the amines, also were prepared for each of the side chains. The structural formula for No. 839, β -(2,5-dimethoxyphenyl)- β -hydroxyisopropylamine is given below as a typical example:



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TABLE 1
Action on mice

COMP NO	ALAKYL CHAIN	N GROUP	PILOMOTOR	EXOPH THALMOS	SALIVATION	OTHER SYMPTOMS	LD ₅₀ mgm/kgm	NO OF MICE	SLOPE	LIMITS OF ERROR AS %
831	β -hydroxyethyl	pri	long	long	long	Depression	131	20	10	87-114
832	OH	sec	long	long	long	Depression	94	35	11	91-110
833		ter	long	long	long	Depression	200	30	32	96-105
834	R-CH-CH ₂ - X	quat	none	none	none	Fine tremors	41	27	26	97-103
839	β -hydroxyisopropyl	pri	long	long	long	Depression	92	40	10	91-109
840	OH	sec	long	long	long	Depression	96	27	20	92-103
841		ter	long	long	long	Depression	131	27	14	91-110
842	R-CH-CH-CH ₃ X	quat	none	brief	none	Fine tremors	22	36	25	94-106
835	β -hydroxypropyl	pri	brief	brief	none	Depression	206	47	36	97-103
836	OH	sec	brief	brief	none	Fine tremors	147	22	11	88-114
837		ter	none	brief	none	Fine tremors	164	24	20	93-107
838	R-C-CH ₂ - CH ₃ X	quat	none	brief	none	Fine tremors	67	39	14	93-107
819	ethyl	pri	long	long	none	Fine tremors	161	27	55	97-103
820	R-CH-CH ₂ -	sec	long	long	none	Coarse tremors	124	30	31	93-106
821		ter	long	brief	none	Coarse tremors	134	69	44	97-103
822	X	quat	brief	brief	none	Fine tremors	49	72	25	97-104
827	isopropyl	pri	brief	brief	none	Depression	135	37	31	96-104
828	R-CH-CH-CH ₃	sec	brief	brief	none	Depression	116	37	20	94-106
829		ter	brief	brief	none	Fine tremors	125	25	53	97-103
830	X	quat	brief	brief	none	Fine tremors	26	34	21	94-106
823	propyl	pri	brief	none	none	Coarse tremors	107	42	23	95-105
824	R-CH-CH ₂ -	sec	brief	none	none	Coarse tremors	98	27	27	95-105
825		ter	brief	brief	none	Coarse tremors	101	32	16	92-108
826	CH ₃ X	quat	brief	brief	none	Fine tremors	36	40	10	90-111

"R" stands for the 2,5 dimethoxy phenyl grouping and "X" for the nitrogen containing group "pri", "sec", "ter", and "quat" are abbreviations for primary, secondary, tertiary and quaternary respectively. The figures in the column headed "Slope" give the slopes of the various log dosage probit response curves. The "Limits of error" give the range within which the LD₅₀ values may be expected to fall 19 out of 20 times. The figures given are expressed as percentages and are to be applied to the corresponding LD₅₀.

Reports on the pharmacological activity of two members of this series have been published previously. Hjort (5) discussed No 820, β -2,5 dimethoxyphenylethylmethylamine and Graham and Cartland (6) described No 823, β -2,5 dimethoxyphenylpropyl amine.

TOXICITY. The LD_{50} values for albino mice were determined by a procedure previously described (7). The precision of these figures is relatively high as is shown by the limits of error in Table 1, but the absolute values may be modified by such factors as environment, diet, etc. (8, 9).

With a few exceptions, the compounds of this series were neither very toxic nor were they innocuous. With respect to chemical structure, the amines were decidedly less toxic than were the corresponding quaternary compounds. The presence of a β -hydroxy group seemed to reduce toxicity in the propylamine group, but was without definite effect in the other two groups. Pilomotor action and exophthalmic effects either were absent or were weak, with the quaternary compounds, and with the propyl and the β -hydroxypropyl chains. Fine tremors generally were observed with these groups. Depression commonly was observed with the β -hydroxy amines, while signs of central nervous stimulation were frequent with the non-hydroxy amines.

As for correlations between pharmacological observations, the more toxic of the amines were also the more potent pressors. The pressors also produced the most pronounced pilomotor actions and exophthalmic effects. The duration of these effects appeared to be proportional to the duration of the pressor action. Salivation, when it occurred, was found with those β -hydroxy compounds which were also pressors. The presence of these three signs, pilomotor action, exophthalmic effects and salivation, usually served to predict pressor properties, and the duration of the phenomena appeared to be directly correlated with the duration of pressor activity. Exceptions to this rule are No. 833 and No. 841.

CIRCULATORY EFFECTS. Circulatory effects were studied in dogs treated with 'Dial'. Blood pressure was measured with a mercury manometer. All drugs were given intravenously. Details of the procedure have been described previously (7).

It may be stated that measurements were reasonably consistent, although numerical estimates of their precision were not obtained. The maximum rise or fall in blood pressure may be readily obtained, but the duration of such effects is difficult to measure when the return of the blood pressure to the initial level is gradual and when sufficient time has elapsed to allow other extraneous factors possibly to affect the level. Heart rates were obtained from blood pressure records. Tachyphylaxis was always found to be associated with prolonged pressor activity. Each drug was tested on at least two, and frequently on more than two, dogs. Results are given in Table 2.

To establish a relative basis for comparison, the dose required to produce a rise of 50 mm. of Hg is given for all pressor compounds, except for 3 of the less potent drugs. Depressor effects were feeble and transient, and for this type of compound the effects produced by a constant dose of 0.008 mM. per kgm. are given in the table.

The outstanding feature of this study was the discovery of several very powerful, long-acting pressor compounds. Four of these, the primary and secondary β -hydroxyethyl derivatives, No. 831 and No. 832, and the primary and secondary β -hydroxyisopropyl compounds, No. 839 and No. 840, were effective for an hour

or more, at doses equal to or less than 0.001 mM. per kgm. No. 839, in particular, is a remarkable drug and will be the subject of a separate communication.

TABLE 2

	PRIMARY	SECONDARY	TERTIARY	QUATERNARY
β -hydroxyethyl	No. 831 0.001 mM/kgm. P50, 60 min. Rate -, T Ep 0, ACh -	No. 832 0.0005 mM/kgm. P50, 60 min. Rate -, T Ep 0, ACh -	No. 833 0.008 mM/kgm. D12, 1 min. Rate 0 Ep +, ACh -	No. 834 0.008 mM/kgm. D18, 2 min. Rate 0 Ep 0, ACh 0
β -hydroxy-isopropyl	No. 839 0.0006 mM/kgm. P50, 120 min. Rate -, T Ep +, ACh 0	No. 840 0.001 mM/kgm. P50, 90 min. Rate -, T Ep +, ACh -	No. 841 0.008 mM/kgm. D16, 1 min. Rate 0 Ep +, ACh -	No. 842 0.008 mM/kgm. D22, 2 min. Rate - Ep +, ACh 0
β -hydroxypropyl	No. 835 0.008 mM/kgm. D24, 1 min. Rate 0 Ep 0, ACh 0	No. 836 0.008 mM/kgm. D15, 1 min. Rate 0 Ep 0, ACh 0	No. 837 0.008 mM/kgm. D5, 1 min. Rate 0 Ep 0, ACh 0	No. 838 0.008 mM/kgm. D25, 5 min. Rate 0 Ep +, ACh 0
ethyl	No. 819 0.0025 mM/kgm. P50, 24 min. Rate -, T Ep 0, ACh 0	No. 820 0.002 mM/kgm. P50, 20 min. Rate -, T Ep -, ACh 0	No. 821 0.003 mM/kgm. P50, 15 min. Rate -, T Ep R, ACh 0	No. 822 0.008 mM/kgm. P30, 5 min. Rate -, T Ep +, ACh 0
isopropyl	No. 827 0.008 mM/kgm. P37, 15 min. Rate -, T Ep 0, ACh 0	No. 828 0.008 mM/kgm. D30, P10, 10 min. Rate -, T Ep -, ACh 0	No. 829 0.008 mM/kgm. D35, 1 min. Rate 0 Ep 0, ACh 0	No. 830 0.008 mM/kgm. D10, 1 min. Rate 0 Ep +, ACh 0
propyl	No. 823 0.008 mM/kgm. D10, 2 min. Rate - Ep 0, ACh 0	No. 824 0.008 mM/kgm. D22, 2 min. Rate 0 Ep 0, ACh 0	No. 825 0.008 mM/kgm. D50, 5 min. Rate - Ep 0, ACh -	No. 826 0.008 mM/kgm. D24, 2 min. Rate - Ep +, ACh 0

"P" stands for an increase in systolic pressure, "D" for a decrease in diastolic pressure and the succeeding numeral indicates the extent of change in mm. of Hg. The duration of the effects are given in minutes. "T" stands for tachyphylaxis. "Rate" stands for heart rate. "Ep" and "ACh" are abbreviations for epinephrine and for acetylcholine, respectively. "+" means enhancement, "-" means diminution, and "0" means no change. "R" indicates a reversal of the pressor action of epinephrine.

Pressor activity was not a universal attribute of this series. Only 10 of the 24 compounds were pressors, while the rest were transient depressors.

With regard to structure and circulatory effects, pressor action, with a few exceptions, was limited to the ethyl and isopropyl primary and secondary amines. Within these limits the presence of a β -hydroxy group enhanced pressor potency and duration. By contrast, pressor properties were never found for compounds containing a β -methyl group; i.e. the propyl compounds. (Graham and Cartland (6) report that β -2,5-dimethoxyphenylpropylamine is a weak pressor.) With the exceptions of the 2 ethyl derivatives, No. 821 and No. 822, the presence of a tertiary or quaternary nitrogen atom also appeared to be adverse to pressor activity.

The heart rate frequently was reduced temporarily. This was always the case with the pressor members of the series, when it might be expected to be associated with the sharp rise in blood pressure, but it was also noted with about one-third of the depressor compounds.

All β -hydroxyisopropyl derivatives enhanced the pressor action of epinephrine. While this may have contributed to the pressor properties of the primary and secondary members, No. 839 and No. 840, it may be pointed out also that many depressors, such as No. 841 and all of the quaternary compounds, which with one exception were depressors, also enhanced the pressor action of epinephrine. Furthermore, No. 821, the tertiary ethyl derivative, combined pressor properties with the ability to reverse the action of epinephrine. Of the remaining members of the series, none had an effect on epinephrine action, except No. 820 and No. 828, which decreased it.

The ability to diminish the depressor action of acetylcholine was shown by the primary, secondary and tertiary β -hydroxyethyl compounds and the secondary and tertiary β -hydroxyisopropyl compounds; No. 839, the primary β -hydroxyisopropyl derivative being the exception. The property was absent from all compounds containing a quaternary nitrogen atom or a β -methyl group or lacking a β -hydroxy group; No. 825, the tertiary propyl derivative, was the single exception.

MYDRIATIC ACTIVITY. Solutions of the drugs were instilled into the conjunctival sacs of albino rabbits. The diameter of the pupil was measured at systematic intervals with a millimeter rule. An X-ray viewing screen furnished a constant source of light. Log concentration-response graphs were plotted and the concentrations that were required to produce 50 per cent increases in pupil diameters in 30 minutes were read from the graphs. This value, which we have called " MC_{50} " (mydriatic concentration₅₀) for the sake of convenience, affords a means for the comparison of relative intensities of action. It was possible to achieve a fair degree of precision, approximately ± 20 per cent, by using 4 rabbits for each of 3 concentration levels. Values were quite reproducible. Species differences were pronounced. Some of the drugs were highly potent in rabbits, but all were ineffective in cats. The results are given in Table 3.

When mydriasis was produced it was prompt in onset, reached a maximum in 15 to 30 minutes and lasted for 2 to 4 hours. According to these experiments, some members of this series were highly potent mydriatics. No. 839, β -(2,5-dimethoxyphenyl)- β -hydroxyisopropylamine, No. 831, β -(2,5-dimethoxy-

phenyl)- β -hydroxyethylamine, and No. 832, β -(2,5-dimethoxyphenyl)- β -hydroxyethylmethylamine, were effective in concentrations as low as 0.02 per cent. Under the same conditions, the MC₅₀ for ephedrine was 4.1 per cent.

With respect to structural relationships, the presence of a β -hydroxy-group generally enhanced activity, but the β -methyl group, i.e., propyl and β -hydroxy-propyl derivatives, was associated with inactive compounds. All but one tertiary compound and all quaternary compounds were inactive.

The correlation between mydriatic action and pressor properties was strong. Mydriatic action was shown only by pressor compounds and all pressor compounds, save the quaternary substance, No. 822, were mydriatics. Mydriatic

TABLE 3
The mydriatic effects on the albino rabbit

ALKYL SIDE-CHAIN	PRIMARY —NH ₂	SECONDARY —NHCH ₃	TERTIARY —N(CH ₃) ₂	QUATERNARY —N(CH ₃) ₂ Cl
β -hydroxyethyl	No. 831 MC50 0.023	No. 832 MC50 0.019	No. 833 MC50 i	No. 834 MC50 i
β -hydroxyisopropyl	No. 839 MC50 0.017	No. 840 MC50 0.054	No. 841 MC50 i	No. 842 MC50 i
β -hydroxypropyl	No. 835 MC50 i	No. 836 MC50 i	No. 837 MC50 i	No. 838 MC50 i
ethyl	No. 819 MC50 0.603	No. 820 MC50 0.347	No. 821 MC50 1.128	No. 822 MC50 i
isopropyl	No. 827 MC50 0.692	No. 828 MC50 i	No. 829 MC50 i	No. 830 MC50 i
propyl	No. 823 MC50 i	No. 824 MC50 i	No. 825 MC50 i	No. 826 MC50 i

"MC50" stands for per cent concentration required to increase diameter of the pupil by 50 per cent.

"i" indicates inactivity.

potency was proportional to pressor potency, and the more potent pressors possessed the greater mydriatic activity. Mydriasis was abolished by 0.5 per cent physostigmine, but pilocarpine was ineffective at 2 per cent levels. It is interesting to note that 3 of the most powerful mydriatics were antagonistic toward the ability of acetylcholine to depress blood pressure (see Table 3). However, the most powerful mydriatic of the group, No. 839, did not inhibit the action of acetylcholine, and 3 tertiary compounds which were inhibitors failed to produce mydriasis. No relationship was apparent between the occurrence of mydriatic properties and the ability of certain members of the group to enhance epinephrine activity.

ACTION ON ISOLATED TISSUE. Table 4 shows the effects of the members of

this series on the tone of smooth muscle (segments of the small intestine and the uterus of virgin adult rabbits, and the whole uterine horn of virgin adult guinea pigs). The sections of tissue were suspended in Van Dyke-Hastings solution (10), which was aerated with a mixture of 94 per cent air and 6 per cent carbon

TABLE 4
Action on isolated smooth muscle

ALKYL SIDE-CHAIN	PRIMARY	SECONDARY	TERTIARY	QUATERNARY
β -hydroxyethyl	No. 831 g.u. 0.03 (+) r.u. 0.03 (+) r.i. 0.03 (-)	No. 832 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)	No. 833 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)	No. 834 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)
β -hydroxyisopropyl	No. 839 g.u. 0.015(+) r.u. 0.015(+) r.i. 0.015(-)	No. 840 g.u. 0.03 (+) r.u. 0.03 (+) r.i. 0.03 (-)	No. 841 g.u. 0.04 (+) r.u. 0.04 (+) r.i. 0.04 (-)	No. 842 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)
β -hydroxypropyl	No. 835 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)	No. 836 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)	No. 837 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)	No. 838 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)
ethyl	No. 819 g.u. 0.015(+) r.u. 0.015(+) r.i. 0.015(-)	No. 820 g.u. 0.015(+) r.u. 0.015(+) r.i. 0.015(-)	No. 821 g.u. 0.015(+) r.u. 0.015(+) r.i. 0.015(-)	No. 822 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)
isopropyl	No. 827 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.06 (-)	No. 828 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.06 (-)	No. 829 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.06 (-)	No. 830 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)
propyl	No. 823 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)	No. 824 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)	No. 825 g.u. 0.06 (+) r.u. 0.06 (+) r.i. 0.06 (-)	No. 826 g.u. 0.12 (+) r.u. 0.12 (+) r.i. 0.12 (-)

"g.u.", "r.u." and "r.i." are abbreviations for guinea pig uterus, rabbit uterus and rabbit intestine respectively. The figures give the average millimolar concentration required to produce a slight stimulation (+), or a slight relaxation (-).

dioxide. The precision of this type of experiment is low and only grossly quantitative results can be obtained.

All 24 members of the group relaxed the intestine and stimulated both kinds of uterine tissue. Some of the compounds were quite active. No. 839, β -(2,5-dimethoxyphenyl)- β -hydroxyisopropylamine, was effective in concentrations as low as 1:333,333. The less potent compounds required concentrations of about 1:33,333. With regard to chemical structure, the only generalization which

could be drawn is that the quaternary compounds almost always were less potent than their homologs.

SUMMARY

A systematic study of a series of 24 β -2,5-dimethoxyphenylalkyl amine and quaternary compounds resulted in the discovery of several powerful, long-acting pressor substances of which No. 839, β -2,5-dimethoxyphenyl- β -isopropylamine, is outstanding.

Pressor activity generally was restricted to the primary and the secondary amines of the ethyl and the isopropyl series. Within these limits, the presence of a β -hydroxy group increased pressor activity. The β -methyl group, as manifested by the propyl compounds, always was inhibitory to pressor action. Tertiary and quaternary nitrogen derivatives usually were inactive. The β -hydroxyisopropylamines and compounds containing quaternary nitrogen enhanced epinephrine activity. The depressor effect of acetylcholine was decreased by certain compounds. All compounds contracted the isolated guinea pig and the isolated rabbit uterus, and all relaxed the isolated rabbit intestine.

Pressor activity was correlated strongly with mydriatic potency. Exophthalmos, salivation and pilomotor effects in the mouse usually, but not always, were associated with pressor properties.

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ANTICONVULSANT ACTION OF ISOPROPYL ALCOHOL

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In preliminary reports (1, 2, 3) it has been indicated that isopropyl alcohol is effective for raising the cortical threshold for electrical convulsions, comparing favorably with diphenylhydantoin and phenobarbital. The acetonemia which accompanies the central depression of isopropyl alcohol is not only suggestive of the possible fundamental nature of this action, but, by the same token, has intriguing possibilities for developing the old ketogenic principle in anticonvulsant therapy. With these general objectives in mind, we have investigated certain relevant actions of this alcohol. This paper reports the details of the acute effects of isopropyl alcohol alone and in combination with drugs currently used in treating epilepsy, and other features of its actions. Subsequent papers are planned to discuss its safeness and other aspects.

METHODS Rabbits, cats and rats were used throughout. Clonic (epileptiform) convulsions were produced by passing an electric current through the brain. The technique used was that described by Tainter and associates (4) used routinely for several years in this department. Formerly, a 10 second period of stimulation was used almost entirely, but it will be explained further on that a shorter period has certain advantages. The stimulation in rats was usually begun with a current of 6 m a., in rabbits, 14 m a., and in cats 20 m a., being repeated at 5 minute intervals and increasing the amount 2 m a. each time until the threshold value was reached. This value remains practically unchanged for the purpose at hand, since it is reproducible within 1 to 2 m a. when determinations are repeated at intervals of several days. Frequent, e.g., consecutive daily stimulations, however, tend to produce somewhat higher thresholds. Therefore, the animals were always allowed to rest 3 to 5 days before a drug was administered and the threshold redetermined.

The drugs used were given gastrically. Food was always withdrawn 1 day prior to medication, and the drug was given 1 hour before redetermination of the threshold. Isopropyl alcohol and tridione were administered as such and phenobarbital and diphenylhydantoin as their sodium salts, all in aqueous solutions. Different animals were used throughout, i.e., no animal receiving a drug was used again for the same or another drug. The difference in the thresholds was taken as a measure of the effectiveness of the drug, and, because of individual variations, had to exceed 10 per cent to be considered significant. With 2 exceptions, the results in the tables and curves represent averages, usually for groups of 10 rats, 5 rabbits, and 3 cats, more cats being unavailable due to a statewide antivivisection campaign.

ISOPROPYL ALCOHOL COMPARED WITH SOME CURRENT ANTIEPILEPSY DRUGS. Table 1 presents results with isopropyl alcohol, diphenylhydantoin, phenobarbital and tridione used individually and in combinations of the alcohol and the other drugs. The dosage of isopropyl alcohol used, i.e., 1250 mg. per kg., was about one-fourth to one-half the anesthetic dose, the doses of the other agents

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TABLE 1

*Isopropyl alcohol and some antiepilepsy agents on thresholds for electrical convulsions**

DRUGS	DOSE	RATS				RABBITS				CATS				REMARKS
		No.	Average		Increase	No.	Average		Increase	No.	Average		Increase	
			B	A			B	A			B	A		
	mg./kg.		m.a.	m.a.	%		m.a.	m.a.	%		m.a.	m.a.	%	
Isopropyl alcohol..	1250	15	8.8	13.2	50	5	20.8	34.4	65	3	32.0	50.7	58	No general depression; no ataxia
Sodium diphenyl hydantoin	100	10	8.4	8.8	5	5	24.8	30.4	22	3	34.7	53.4	53	No general depression; no ataxia
Sodium phenobarbital...	50	10	8.6	10.4	21	5	25.6	38.0	48	3	22.0	47.2	115	Rats: slight motor depression. Rabbits: moderate depression. Cats: marked depression & ataxia
Tridione.....	100	10	8.0	8.4	5	5	20.0	22.0	10	3	32.0	38.6	20	No general depression; no ataxia
Isopropyl alcohol..	1250	10	6.8	11.2	65	8	21.2	37.5	77	8	30.0	80.0	167	No general depression; no ataxia
plus sodium diphenylhydantoin	100													
Isopropyl alcohol..	1250	10	7.2	14.0	94	5	19.8	37.4	89	3	25.3	76.0	200	Marked general depression and ataxia
plus sodium phenobarbital	50													
Isopropyl alcohol..	1250	10	6.8	9.4	40	5	18.4	27.6	50	3	27.0	51.0	89	Slight depression & ataxia
plus tridione...	100													

*Stimulations were made for 10 seconds in all animals before, and 1 hour after, gastric administration of drugs. In the table, m.a. = milliamperes; No. = number; B = before, A = After.

being those commonly employed in tests for effects on the cortical threshold. A 10-second stimulation was used in these tests.

It is seen that isopropyl alcohol (1250 mg. per kg.) alone was more depressant in all 3 species used than diphenylhydantoin and tridione, but only in rabbits and rats more effective than phenobarbital, which was more effective in cats. The increase in cortical threshold after isopropyl alcohol ranged from 50 to 65 per cent, whereas the increases after diphenylhydantoin (100 mg. per kg.) were only from 5 to 53 per cent, and after tridione (100 mg. per kg.) 5 to 20 per cent. The increases after phenobarbital in doses of 50 mg. per kg., the smallest of all doses used, ranged from 21 to 115 per cent with evidences of motor depression in all species. The ataxia was least marked in rats and greatest in cats whose cortical threshold was raised the most. On the other hand, the uniformly high increases in thresholds after isopropyl alcohol were obtained without demonstrable ataxia and other manifestations of general depression. The latter was true also for diphenylhydantoin and tridione whose threshold raising powers were definitely less, or negligible, in the dosage used in all animals except cats in which the depressant effect was only definite after diphenylhydantoin. Therefore, isopropyl alcohol compared favorably with diphenylhydantoin as to selectivity of the central depression, being more purely anticonvulsant. The alcohol was definitely superior to phenobarbital, since its anticonvulsant action was produced without generalized motor depression. All animals receiving isopropyl alcohol were somewhat quieter than normals without medication, but their reflexes and voluntary locomotion were preserved. All recovered uneventfully after the single doses used.

ISOPROPYL ALCOHOL COMBINED WITH SOME ANTIEPILEPSY AGENTS. The same doses of isopropyl alcohol and of diphenylhydantoin, phenobarbital and tridione were given together to rats, rabbits and cats as of the same drugs used alone. The results in Table 1 show that a summation or better of the per cent increases in cortical thresholds occurred when the alcohol and diphenylhydantoin or phenobarbital were administered together to all animals except rabbits in which the changes were close to or less than summation. Tridione with isopropyl alcohol produced smaller increases in thresholds than the alcohol alone in rats (40 per cent) and rabbits (50 per cent), there being only little more than summation of effects in cats (89 per cent). An outstanding result was the considerable increase in cortical threshold with only the combination of isopropyl alcohol and diphenylhydantoin which occurred without demonstrable motor depression or ataxia. The combination with phenobarbital caused marked ataxia and there was motor depression with the tridione combination.

ANTICONVULSANT EFFICIENCY OF ISOPROPYL ALCOHOL ACCORDING TO TWO DIFFERENT PERIODS OF STIMULATION. Cyanosis of the mucosae in rodents especially was suggestive at different times when these animals had been stimulated for 10-second periods. Cerebral anoxia could conceivably raise or lower the cortical threshold and thus decrease or increase, or prematurely initiate, the clonic convulsions, a possible complication which required clarification before extending our results. Furthermore, while our work was in progress, Barany

and Stein-Jensen (5) using rabbits reported that shortening the period of electrical stimulation to somewhat more than $\frac{1}{2}$ (from 1.0 to 0.21 second) by their method raised the cortical threshold appreciably (about twice), similar or somewhat greater differences between their short and long stimulations being observed after giving certain barbiturates and diphenylhydantoin. Therefore, it seemed desirable to compare different stimulating periods in medicated and unmedicated animals.

We compared stimulating periods of 2 and 10 seconds in unmedicated rats, and in rats, rabbits and cats receiving isopropyl alcohol. In 25 unmedicated rats, the ranges for the 2 periods of stimulation, with adequate rest-periods between stimulations, were nearly the same, i.e., 6 to 10 m.a. (10-second stimulation) and 6 to 12 m.a. (2-second stimulation) with a median increase of only 2 m.a., or 17.5 per cent, for the 2-second stimulation. Since differences of ± 10.0 per cent fall within range of individual variations and of experimental

TABLE 2

*Cortical threshold changes according to two different periods of electrical stimulation and two different doses of isopropyl alcohol**

DOSE	STIMULATION PERIOD	NO.	RATS AVERAGE M.A.		INCREASE	NO.	RABBITS AVERAGE M.A.		INCREASE	NO.	CATS AVERAGE M.A.		INCREASE
			B	A			B	A			B	A	
mg./kg.	sec.				%				%				%
500	10	10	9.8	10.8	10	5	17.6	20.0	12	3	35.0	43.0	23
	2	10	8.4	9.6	14	5	22.0	28.8	27	3	33.2	42.6	28
1,250	10	15	8.8	13.2	50	5	20.8	34.4	65	3	32.0	50.7	58
	2	10	9.6	14.2	48	5	23.0	36.3	58	3	38.7	62.7	60

* Stimulations were made before, and 1 hour after, gastric administration of the alcohol. m.a. = milliamperes; sec. = seconds; B = before; A = after.

error, the net increase of 7.5 per cent favored to a minor extent the shorter period of stimulation in these unmedicated rats.

The results presented in Table 2 were obtained with 2 different doses of isopropyl alcohol, i.e., 500 and 1250 mg. per kg., in rats, rabbits and cats, using 10, 5 and 3 animals for each dose, respectively. The great majority of the trials, or 5 of 6, with the 3 species and the 2 doses of the alcohol used showed changes of only from -7 to +5 per cent. Only the rabbits given the 500 cc. dose of the alcohol showed an increase of 15 per cent for the 2-second stimulation, a minor increase considering the small number of rabbits used. Collectively, therefore, our results indicated practically no differences between the short and longer periods of stimulation as to per cent changes in cortical thresholds in 3 different species receiving isopropyl alcohol gastrically, although the average cortical thresholds were variably increased in nearly all. There was, of course, the usual greater effectiveness of the higher dose of the alcohol in all animals. There was no evident cyanosis with the 2-second period and the fact that the results with

the 2 stimulations were practically identical justified considering the results obtained with the longer period together with those with the shorter one. The 2-second period was used in the majority of the remaining experiments and has become the preferred routine procedure.

ANTICONVULSANT EFFICIENCY ACCORDING TO DOSAGE OF ISOPROPYL ALCOHOL. Seven doses of isopropyl alcohol ranging from 250 to 4000 mg. per kg., were tried on 3 different species with the object of determining their graded effectiveness on the cortical thresholds, general symptoms, and safeness of this agent.

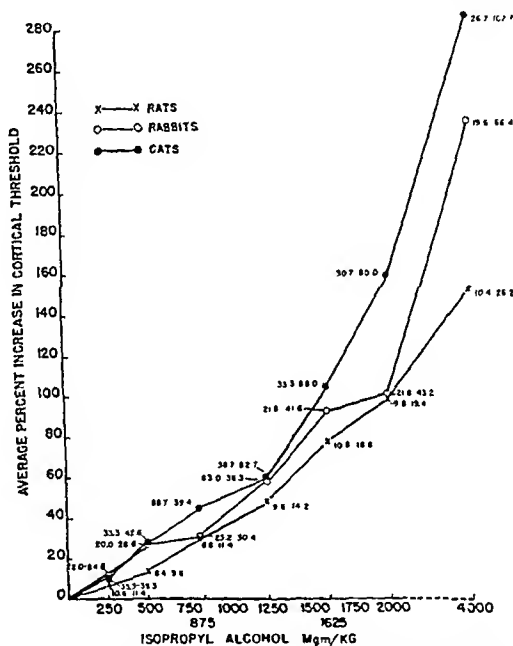


FIG. 1. ANTICONVULSANT EFFICIENCY ACCORDING TO DOSAGE OF ISOPROPYL ALCOHOL IN RATS, RABBITS AND CATS

Electrical stimulations were made for 2 seconds before, and 1 hour after, gastric administration of each dose of the alcohol; averages of the thresholds in m.a., before and after each dose of the alcohol are indicated on the curves. For each dose there were used 10 rats, 5 rabbits and 3 cats.

The alcohol was given gastrically using for each dose 10 rats, 5 rabbits and 3 cats. The averages for the thresholds before and after the alcohol and the per cent changes for the different doses and animals are presented graphically in Figure 1.

It is seen that doses of from 500 to 4000 mg. per kg. were definitely effective in raising the cortical thresholds (2-second stimulations) in all 3 species used, the changes ranging from averages of about 25 to 300 per cent. The changes after 250 mg. per kg. fell within range of individual variations. The highest effective

dose without evidences of motor depression and ataxia was 1250 mg. per kg. Moderate (rats) to marked depression and ataxia (rabbits and cats) was caused by 1625 mg. per kg., severe depression and ataxia in all animals and 1 death (rabbit) after 2000 mg. and severe depression and ataxia in all animals with death in the majority of cats and rabbits after 4000 mg. per kg. All animals receiving 1250 mg. per kg. or less recovered uneventfully and remained in good health. There were no evidences of nausea and vomiting in any of the cats at any time. Single doses of 1250 mg. per kg., may, therefore, be regarded as the highest effective, without apparent undesirable side-effects, and also the safest under the conditions. Repeated doses are to be tested later on.

A group of 30 rats receiving the same range of doses of the isopropyl alcohol gave practically the same average per cent changes after each dose and 10-second

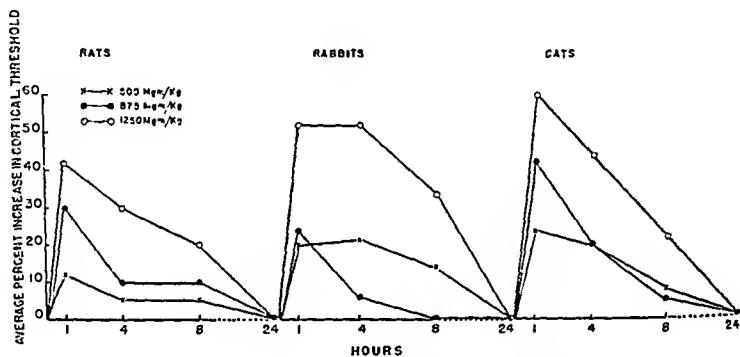


FIG. 2. DURATION OF ANTICONVULSANT EFFECTS OF THREE DIFFERENT DOSES OF ISOPROPYL ALCOHOL IN RATS, RABBITS AND CATS

Thresholds were determined with 2-second stimulations before, and at the different periods after, gastric administration of the alcohol in each animal. Per cent changes in m.a., represent averages of 5 rats, 3 rabbits and 3 cats for each dose.

periods of stimulation. Motor depression occurred only after the high doses of 2000 and 4000 mg. per kg.

DURATION OF ANTICONVULSANT ACTION. The duration of the anticonvulsant action of isopropyl alcohol was determined according to cortical threshold changes (2-second stimulations) at end of 1, 4, 8 and 24 hours after giving 3 different single doses each to groups of 5 rats, 3 rabbits and 3 cats. The results are presented graphically in Figure 2 which shows the average per cent increases in m.a. at each time-period for each dosage and each animal species. The average changes, and the grand averages in parenthesis, for the control m.a.'s, before the alcohol, for the different groups of animals on all doses were as follows: 8.4-10.4 (8.9) 15 rats; 22.7-24.0 (23.3) 9 rabbits; 23.3-38.7 (32.8) 9 cats.

It is quite clear that the peak of the cortical depression was generally reached in 1 hour after administration of the alcohol in all doses in all animals, thus suggesting a fairly rapid absorption and central action. The depression was definitely less at end of 4 hours after all doses in cats and rats, but in rabbits

remained about the same as at the end of 1 hour. In 8 hours, the recovery from the peak effects was at least $\frac{1}{2}$ to $\frac{2}{3}$ and even more, according to amperage, in the great majority of animals, and recovery was complete at end of 24 hours in all animals on all doses.

In general, the order of anticonvulsant efficiency of all doses of the alcohol at the different testing periods are about as follows: rats < rabbits < cats (most responsive to drug). For instance, the optimal dose of 1250 mg. per kg., gave the following peak increases in thresholds for the different species: 44 per cent,

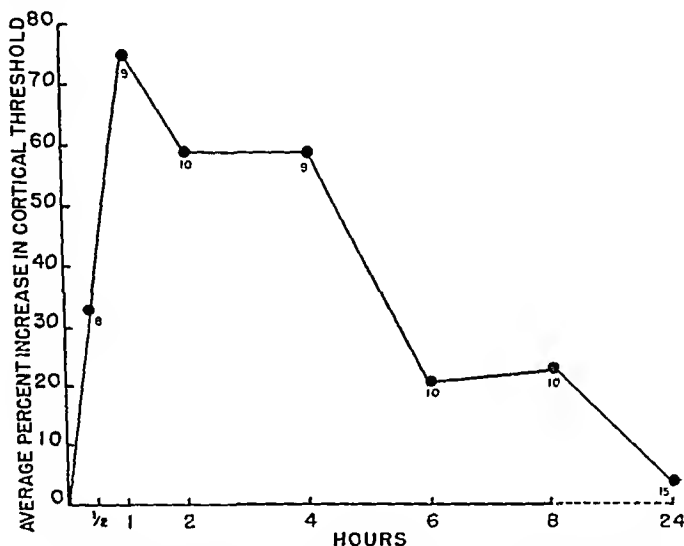


FIG. 3. DURATION OF ANTICONVULSANT EFFECT OF 1250 MG. PER KG. OF ISOPROPYL ALCOHOL ADMINISTERED GASTRICALLY IN RATS

Thresholds were determined with 10-second stimulations before, and at the different periods after, the alcohol in each animal. Per cent changes in m.a., represent averages of groups of rats, the number of rats for each dose being indicated by the numerals on the curve.

rats; 54 per cent, rabbits; and 60 per cent, cats. Other doses produced similar patterns of graded increases in the different species.

Additional data were obtained on 71 rats, using 10-second stimulations, at intervals of from $\frac{1}{2}$ hour to 24 hours after gastric administration of the alcohol, with essentially the same results as to peak and duration of the depression as in the other animals stimulated for 2 seconds. The results are shown in Figure 3, which leaves no doubt that the peak occurred in 1 hour with some recovery in 2 and 4 hours which was greatly advanced in 8 hours and complete in 24 hours.

ACETONEMIA AFTER ISOPROPYL ALCOHOL. The metabolic fate of isopropyl alcohol has long been known to be oxidation to acetone, which has been detected in the blood and urine. The central nervous depression of this secondary alcohol

is generally attributed to the acetoneemia which might be related quantitatively to the changes in cortical threshold and thus be a determining factor in the anti-convulsant efficiency of the alcohol. If the degree of acetoneemia and changes in threshold efficiency ran parallel, this would be suggestive of cause and effect. Therefore, the blood acetone was determined in cats and rabbits receiving 2 different doses of isopropyl alcohol gastrically. Ten rabbits were given 500 mg. each, and 3, 1250 mg. per kg. each; 6 cats, 3 each, received doses of 500 and 1250 mg. per kg. Blood acetone was estimated according to the micromethod of Greenberg and Lester (6) at intervals of 1, 4, 8 and 24 hours after giving the alcohol and the cortical thresholds were determined with 2-second stimulations

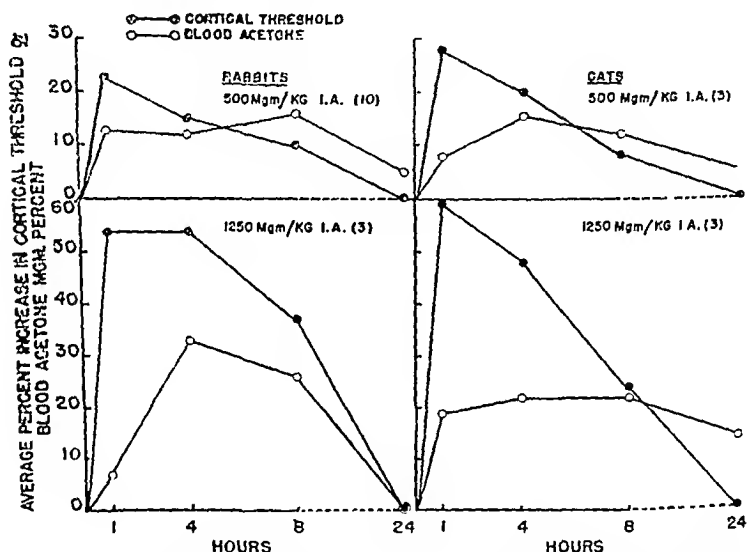


FIG. 4. CORTICAL THRESHOLD CHANGES AND ACETONEMIA AFTER TWO DIFFERENT DOSES OF ISOPROPYL ALCOHOL GASTRICALLY IN RABBITS AND CATS
I. A. = isopropyl alcohol; numerals in parenthesis, number of animals used.

at the same periods. In the rabbits the cortical thresholds were determined at once after removing blood from a leg vein for acetone, but in the cats the thresholds and blood acetone were determined separately on 2 different occasions. The results are presented in Figure 4, which shows the cortical thresholds as average per cent increases over the controls and the blood acetone as average net increases after deducting the average control or normal values, which were 22.0 mg. per cent for the cats and 18.0 mg. per cent for the rabbits. The average absolute m.a.'s, have been omitted from the curves, but were of the same general order of magnitude as in all previous experiments with the doses used.

Only approximate comparisons can be made, since the number of animals on the different doses of the alcohol was not the same, and the number of cats was

small. In general, however, the degree of acetonemia in the 2 species on corresponding doses was of the same general order of magnitude. The blood acetone content after the smaller dose of 500 mg. per kg. was roughly $\frac{1}{2}$ or $\frac{1}{3}$ that after the higher dose of 1250 mg. per kg., generally at 4 hours and 8 hours. At 24 hours, the content varied considerably, but, in the majority of animals, was at the zero level, or not more than 5 mg. per cent above normal, except after 1250 mg. in cats where it was 14 mg. per cent. The peak of cortical depression as indicated by the average per cent changes in the thresholds of the majority of animals (except rabbits on 500 mg. at 8 hours) occurred in 1 hour after administration of the alcohol, preceding the peak of the acetonemia which occurred at 4 hours in practically all except rabbits on 500 mg. where it occurred at 8 hours. The 2 peaks, therefore, did not coincide, or it can be said that the blood acetone kept on increasing when the cortical thresholds were decreasing. In other words, our data show a lack of correlation of the suggested cause (optimal acetonemia) and effect (optimal cortical depression). However, the forms of the curves for the data on acetonemia and cortical threshold changes showed a certain parallelism at each level which leaves the possibility that blood acetone and central depression may be intimately related, although, of course, this does not eliminate a possible depressant action of the alcohol itself before it is effectively metabolized to acetone. Our conditions did not permit making alcohol estimations at the same time, but these, and blood acetone determinations between the 1-hour and 4-hour periods, are planned.

The degree of acetonemia in 2 other and larger groups of rabbits without simultaneous cortical stimulations was higher than in the rabbits of Figure 4 with cortical stimulations. For instance, in 15 rabbits given 500 mg. per kg. of the alcohol gastrically the average increases in mg. per cent of blood acetone were as follows: 21 (1 hour), 20 (4 hours), 16 (8 hours) and 6 (24 hours). In 10 rabbits receiving gastrically 1250 mg. per kg., the average increases in mg. per cent of acetone were: 15 (1 hour), 30 (4 hours), 34 (8 hours) and 15 (24 hours). Whether the electrical stimulation itself could affect blood acetone was not determined, but these and other features require further examination before a correlation of acetonemia and cortical depression may be established.

DISCUSSION. The results of this study of isopropyl alcohol as an anticonvulsant in 3 different species of about 477 animals leave no doubt that it compares favorably with diphenylhydantoin under the similar conditions, except that dosage is much higher which makes the alcohol weaker. It is less effective than phenobarbital, whose anticonvulsant action is unobtainable without generalized motor depression and ataxia, which are lacking with both isopropyl alcohol and diphenylhydantoin. Isopropyl alcohol appears superior to tridione. When isopropyl alcohol and diphenylhydantoin are given together the action is definitely increased, being somewhat greater than summation of their cortical thresholds and without general motor depression and ataxia. Given with phenobarbital, however, the ataxia and motor depression are severe, even when tried with one-half the dosage of phenobarbital. The effects are greater than summation of the thresholds. Not more than summation is gained when the

alcohol is given with tridione. Comparison of these drugs for anticonvulsant efficiency is precluded due to differences in dosage used.

The optimal dosage of isopropyl alcohol for raising the threshold without other effects seems high, i.e., 1250 mg. per kg., or roughly $\frac{1}{4}$ to $\frac{1}{2}$ the narcotic dose, and is much higher than the dosage of diphenylhydantoin (100 mg. per kg.) or phenobarbital (50 mg. per kg.). However, all animals on this dosage recovered uneventfully and remained in good condition for months. Much higher doses (2000 mg. and over per kg.) caused deaths in the majority of animals several days after administration. Keith (7) found 0.5 cc. per kg., intravenously of both the isopropyl and propyl alcohols rather toxic in rabbits and without anticonvulsive effect on thujone convulsions which may run a different course than the electrical, and intravenous injections may result in rapid elimination, before adequate acetonemia occurs.

Isopropyl alcohol is not prohibited for internal use by federal law but is not potable and is more toxic than ethyl alcohol, particularly at higher dosage levels, such as the narcotic or fatal. For instance, Lehman and Chase (8) found that, according to the gastric LD_{50} per kg. in rats, rabbits and dogs (6.73, 6.41 and 6.15 cc., or 5.38, 5.13 and 4.92 gm., respectively) isopropyl alcohol was once again as toxic as ethyl alcohol (12.96, 10.22 and 12.25 cc., or 10.86, 8.17 and 9.80 gm., respectively) but the intravenous anesthetic doses of both alcohols for rabbits and dogs were about the same, i.e., 3.23 and 3.35 cc., or 2.58 and 2.68 gm., respectively. Obviously, the optimum single gastric dose for raising the cortical threshold is much less than any of these doses and our results indicate its relative safeness in animals.

However, continued administration of isopropyl alcohol might be unsafe, because, in a limited survey, we found that voluntary drinking by rats of this alcohol for nearly 3 months caused decreases in body weight and growth and some deaths, but without demonstrable pathological tissue changes. It is not certain at this time whether the general effects were caused by the alcohol or decreased food consumption, but further experiments with continued administration are in progress. On the other hand, Harris (9), Boughton (10) and Lehman and Chase (8), who have investigated the possible hazards of giving repeated isopropyl alcohol orally to animals for relatively long periods, concluded that this alcohol was comparatively safe, being only slightly more toxic than ethyl alcohol whose effects it resembles. Even if isopropyl alcohol were safe for man, it does not follow that this alcohol would be any more desirable than ethyl alcohol as a therapeutic anticonvulsant, and it is not to be recommended for clinical trial. This applies also to any derivatives of it or other ketogens until found to be safe by acceptable evidence.

Our results confirm the ketogenic property of isopropyl alcohol reported by others (11). Acute toxicity is sometimes attributed to the acetone formed, but, except for the well known symptoms of hyperketonemia or ketosis, it is not clear in what way the acetone is responsible for the toxicity. Lehman and Chase (8) observed no evidences of delayed toxic effects. That there might be a beneficial side to a moderate grade of acetonemia is generally overlooked in considerations

of its relationship to the actions of isopropyl alcohol, but condoned in the ketogenic diets for epileptics. The following published (1, 2) and unpublished evidences obtained by us may be cited in support of hyperketonemia as being the cardinal feature of the cortical depression of isopropyl alcohol: simple primary alcohols, i.e., ethyl alcohol and n-propylalcohol, are not comparable in anticonvulsant efficiency and do not produce acetone; acetone is effective though fleeting in action and acetone is variable due to rapid elimination; isopropylamine decreases the cortical threshold and is a convulsant, evidence of acetone being lacking. Depressant efficiency, therefore, is favored by a comparatively slowly metabolizable secondary alcohol, i.e., isopropyl alcohol. Tests with a considerable number of complex ketogens did not produce a high order of anticonvulsant efficiency, isopropyl alcohol remaining thus far the most efficient and least objectionable (1).

CONCLUSIONS

1. Isopropyl alcohol given gastrically was found to be a definite anticonvulsant against clonic (epileptiform) convulsions produced electrically, comparing favorably with diphenylhydantoin and being less objectionable than phenobarbital and superior to tridione in the doses used.

2. High anticonvulsant efficiency was preserved, without motor depression and ataxia, when isopropyl alcohol was given together with diphenylhydantoin, but not when given with phenobarbital or tridione which resulted in marked to moderate motor depression. The effects on cortical thresholds of these drug combinations were somewhat better than summation of the effects of the individual drugs in the majority of animals.

3. Isopropyl alcohol alone or in combination with diphenylhydantoin was comparatively safe in the single optimum doses used, or 1250 mg. per kg., all animals recovered uneventfully and survived indefinitely. Higher doses were toxic. The safeness of repeated doses is being investigated.

4. Isopropyl alcohol caused a definite acetone which is believed to be largely responsible for, or intimately related to, its anticonvulsant action, although the average peak changes in blood acetone and cortical thresholds failed to show an exact coincidence. The maximum of cortical depression occurred in 1 hour after administration of the alcohol, but of blood acetone in 2 to 4 hours.

5. The anticonvulsant efficiency of isopropyl alcohol, as a ketogen, is consistent with the well known ketogenic principle in therapy of epilepsy, and is believed to offer a promising lead to improving old or developing new anticonvulsant compounds which might result in more desirable and practicable medication over the use of the generally objectionable ketogenic diets. Thus far isopropyl alcohol has been found to be the most efficient and least objectionable of a considerable number of ketogenic compounds tested.

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GOITROGENIC COMPOUNDS: PHARMACOLOGICAL AND PATHOLOGICAL EFFECTS

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The relation of chemical structure to the goitrogenic action of various chemical compounds has been investigated by many workers. In the course of similar studies we observed that the administration of appropriate doses of thiouracil and 2-aminothiazole resulted in hyperplasia of the thyroid gland without remarkable changes elsewhere. However, other substances, such as bis-(4-acetaminophenyl) selenium dihydroxide produced not only thyroid hyperplasia but also general changes such as retardation and arrest in growth of the animals, atrophy of the thymus and other lymphatic structures, degeneration of various excretory and endocrine glands, and in many instances degeneration of the thyroid gland as well. Many compounds devoid of goitrogenic action also produced these general changes. As the latter have received little attention, they are being presented in this paper in detail. Also, evidence is presented that the thymus is a very sensitive indicator of general disturbances.

MATERIALS AND METHODS. The compounds that were studied are classified according to Astwood's scheme (2). We also included some selenium and silicon analogues of these with known goitrogenic action (3).

1. *Aliphatic compounds containing nitrogen and sulfur, used because of the known activity of thiourea:* β -aminoethyl- β' -carboxyethyl sulfide; β -isothioureia propionic acid; disodium ethylene bis-dithiocarbamate (Dithane); monoacetyl thiourea; diisopropyl xanthogen; diacetyl thiourea; methionine sulfoxide; N-(1,3-dimethyl- Δ^2 -butenylidene)-N'-phenylisothioureia; ethylene thiourea. Sodium sulfite and sodium sulfide were also included in this group.

2. *Cyclic compounds containing sulfur, studied because of the known goitrogenic action of thiouracil:* 2-thiohydantoin-5-propiolactam; 2-thiohydantoin-5-propionic acid; 2-thiohydantoin-5-acetic acid; pseudo-thiohydantoin; 2-mercapto-3-(o-tolyl)-4,6,6-trimethyl hydroypyrimidine; 2-mercapto-3-(p-tolyl)-4,6,6-trimethyl hydroypyrimidine; 2-mercapto-3-cthanol-4,6,6-trimethyl hydroypyrimidine; 2-mercapto-3-alphanaphthol-4,6,6-trimethyl hydroypyrimidine; 2-mercapto-4,6,6-trimethyl hydroypyrimidine; 2-mercapto-3-phenyl-4,6,6-trimethyl hydroypyrimidine; 1,3-diaminophenyl bis-(2-mercapto-4,6,6-trimethyl hydroypyrimidine); 2-mercapto-4-ketodihydrothiazine; 4-ethyl thiazyl disulfide; 4,5-dimethyl thiazyl disulfide; 4,5-dimethyl mercaptothiazole; 2-aminothiazole; acetyl 2-aminothiazole; thiobarbituric acid; thiouracil; isonitroso thioharbituric acid; thiophthalic acid; thialdine; thiamine; benzal thiobarbituric acid; thiophthalic anhydride.

3. *Aminophenyl compounds, studied because simple aromatic amino compounds have goitrogenic action:* p-dimethylaminoazobenzene; p,p'-diaminobenzophenone; aminopyrine; antipyrine; p-phenylenediamine; p-aminobenzoic acid; arsanilic acid; trinitro-diiodo-tyrosine.

4. *Pyridine compounds, used because of their analogy to p-aminobenzoic acid or because of*

a cyano grouping, both of which are known to have goitrogenic action: 2,4-diketo-1,2,3,4-tetrahydro-5,6-(2',3'-pyrido)-pyrimidine; pyridine betaine carboxylic acid; isonicotinic acid; 2-aminonicotinic acid; 2-aminopyridine. Also included in this group are seventeen 2-pyridone derivatives in which the 2, 3, 4, and 5 positions have been variously substituted with carboxy, cyano, nitro, amino, and halogen groups. None of these showed any goitrogenic activity.

5. *Selenium and silicon analogues*: bis-4-acetaminophenyl selenide; selenium dioxide; sodium selenite; sodium selenate; bis-(p-methoxyphenyl) selenium dihydroxide; bis-(4-acetaminophenyl) selenium dihydroxide; bis-(p-methoxyphenyl) selenium dichloride; bis-(4-methoxyphenyl) selenide; diphenylsilanediol; selenium amorphous precipitate.

All experiments were performed on weanling white rats, usually females weighing 30 to 40 grams. The animals were kept in air conditioned quarters maintained at 72°F. Each compound was administered along with a standard laboratory diet to at least four rats for a period of 8 to 10 days. Rats maintained on an unadulterated laboratory diet served as normal controls, while rats receiving 0.05% thiouracil or 0.1% 2-aminothiazole served as goitrogenic controls. The rats were sacrificed under ether anesthesia.

In most experiments the thyroid glands were weighed, and in many instances their iodine content was determined by a modification of Kendall's method (4). In all cases at least one set of thyroid glands was studied microscopically and the goitrogenic action graded according to the degrees of epithelial hyperplasia, follicular enlargement, and loss of colloid from the follicles (Fig. 1). Heart, lungs, liver, kidneys, adrenals, thymus, spleen, lymph nodes, pancreas, salivary glands, pituitary gland, and bone marrow were also examined microscopically. Most of the sections were stained with hematoxylin-eosin, and others with azur II-eosin.

The weight of the thyroid gland, its iodine content, and its microscopic appearance were found to be equally useful criteria of goitrogenic action (Figs. 2 and 3). The weights of normal thyroid glands were less than 200 micrograms/gram of body weight (Figs. 2 and 3). In one series of 25 groups comprising 100 normal rats, the average thyroid weight was 130 micrograms/gram, and in another series of 10 groups comprising 40 rats it was 140 micrograms/gram (Table I). Weights of more than 200 micrograms of thyroid gland per gram of body weight were therefore considered abnormal.

The iodine content of normal thyroid glands averaged 0.17% in 25 groups comprising 100 normal rats (Table I). The lowest values found in these rats were 0.06% in one case and 0.07% in two cases (Fig. 2). Consequently, values of 0.05% and less were considered abnormal.

The histologic appearance of normal thyroid glands was fairly uniform except for grades I and II hyperplasia occasionally observed in control animals. Only two instances more severe than grade I occurred in 140 normal rats, and therefore hyperplasia greater than II was considered abnormal. The hyperplasia observed in these rather acute experiments was macrofollicular (Table II). It will be shown elsewhere that prolonged administration of goitrogenic compounds eventually causes a microfollicular goiter. These observations have considerable bearing on the puzzling origin of the microfollicular hyperplasia occasionally seen in human goiters.

Thyroid weights of 200 micrograms/gram or more, iodine contents of 0.05% or less, and hyperplasia greater than II were found in animals that received thiouracil, 0.01 to 0.05%; propylthiouracil, 0.01 to 0.25%; 2-aminothiazole, 0.001 to 0.2%; aminothiazole sulfate, 0.05

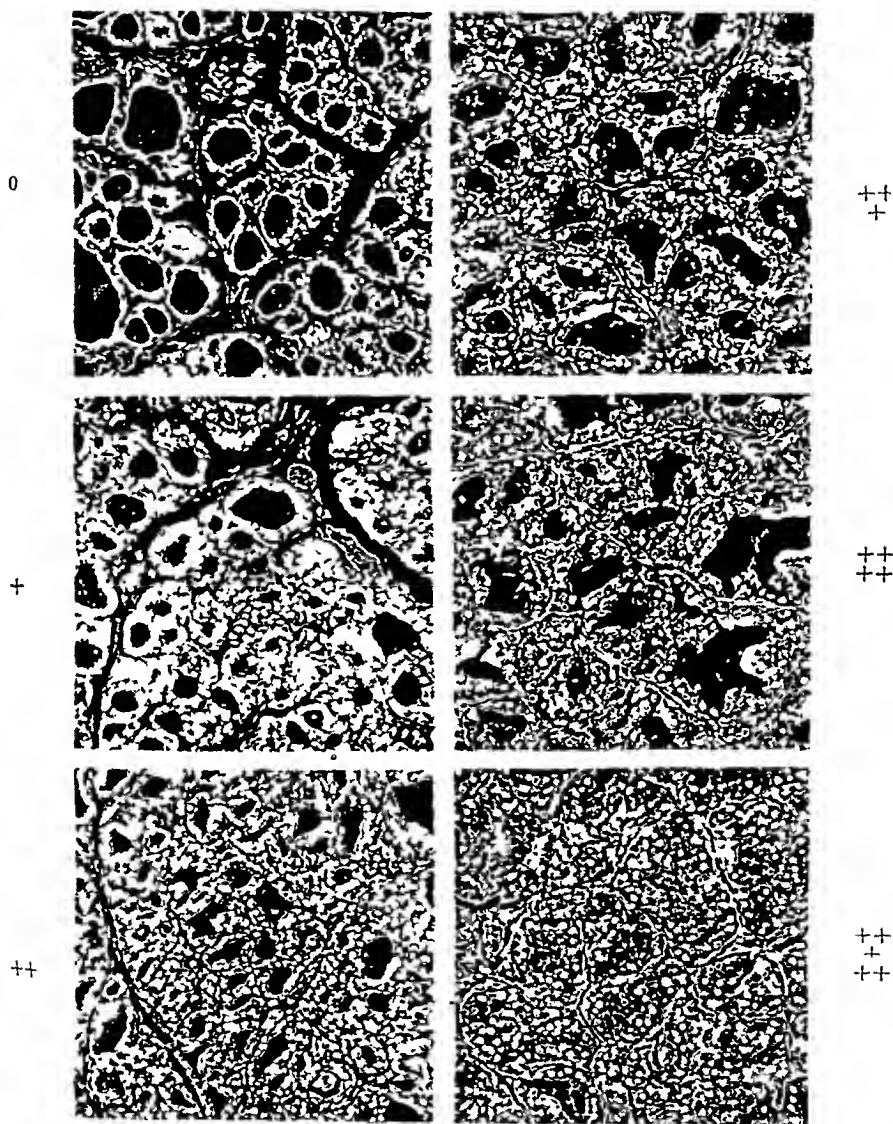


FIG 1 DEGREES OF EPITHELIAL HYPERPLASIA, FOLLICULAR ENLARGEMENT, AND LOSS OF COLLOID FROM THE FOLLICLES

Grades 0, + and ++ may all be found in control animals, grades +++, +++++ and ++++++ were found with active goitrogenic compounds only. Hematoxylin-eosin, X 218

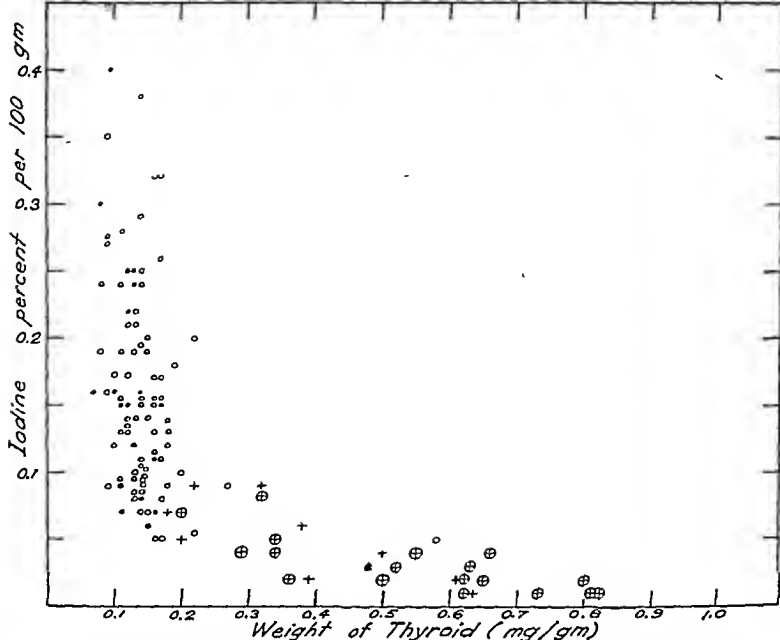


FIG. 2. DECREASE IN IODINE CONCENTRATION (% PER 100 GRAMS OF THYROID TISSUE) WITH INCREASE IN WEIGHT OF THYROID GLAND (MG. PER GRAM OF BODY WEIGHT)
 ● normal controls; ⊕ thiouracil controls; + 2-aminothiazole controls; ○ other compounds tested.

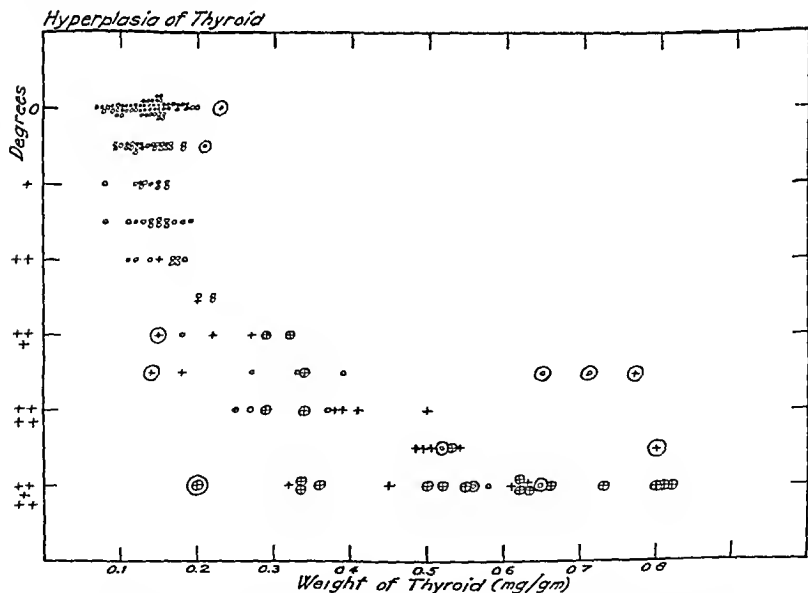


FIG. 3. WEIGHT (MG. PER GRAM OF BODY WEIGHT) AND HYPERPLASIA OF THYROID GLAND (DEGREES)
 ● normal controls; ⊕ thiouracil controls; + 2-aminothiazole controls; ⊙ propylthiouracil controls; ○ other compounds tested.

TABLE I

Local and general effects of active goitrogenic compounds

MATERIAL	NUMBER OF ANIMALS*	DURATION OF EXPERIMENT	WEIGHT OF ANIMALS % CHANGE		THYROID			THYMUS	
			Per day	Of normal	Average weight	I ₂	Hyperplasia	Average weight	Microscopic changes, wasting†

Series I									
	%	days			mg/gm	%	degree	mg/gm	
Normal controls		27/28	10	+6.8	±0	—	—	—	Some advanced 3/27
Thiouracil	0.2	23/24	11	+7.1	+9	—	—	—	0
"	0.5	23/24	11	+6.8	+3	—	—	—	Some advanced 3/23
Monoacetyl thiourea	10	2/4	15	+1.9	-78	—	—	—	Some marked all 0
Bis (4 acetaminophenyl) selenide	0.5	4/4	9	+3.3	-34	—	—	—	—
PABA Bis (4 acetamino phenyl)selenide	10	4/4	9	+7	-91	—	—	—	Some complete all

Series II									
Normal controls		100/100	9	+5.8	±0	13	17	0+	Marked 2/100
Thiouracil	0.5	91/92	9	+5.8	+3	58	03	+++++	Some advanced 3/92
2 Aminothiazole	0.01	4/4	11	+5.0	-14	20	05	+++++	0
"	0.25	4/4	11	+5.3	-9	18	07	+++++	0
"	0.05	4/4	11	+4.4	-24	39	02	+++++	0
"	0.10	12/12	10	+4.1	-26	44	25	+++++	0
"	20	8/8	10	+2.2	-53	56	03	+++++	0
2 Aminothiazole HCl	0.25	4/4	10	+5.4	-15	—	—	+++++	0
"	0.5	8/8	10	+4.8	±0	22	09	+++++	0
Benzal thiobarbituric	2	4/4	9	+4.0	-15	20	10	++ +++	0
Acetyl 2 aminothiazole	25	4/4	9	+2.3	-63	25	—	+++++	Some
di amino benzophenone	25	3/4	—	—	—	—	—	++ +++	Some advanced all
2 Mercapto-3 phenyl 4,6,6 trimethyl hydro pyrimidine	25	4/4	10	+3.6	-43	22	06	+++++	0
p Phenylendiamine	25	4/4	10	-3	-105	—	—	+++++	Some
Dithane	25	4/4	10	+4.4	-8	58	05	+++++	0

Series III									
Normal controls		40/40	9	+6.1	±0	14	—	0	0
Thiouracil	0.1	4/4	10	+8.5	-3	29	—	+++	3.3
"	0.2	4/4	10	+8.2	-7	34	—	+++++	3.25
"	0.5	8/8	10	+6.6	-25	50	—	+++++	2.8
Propyl thiouracil	0.1	3/4	10	+7.2	-18	63	—	+++++	2.9
"	0.5	4/4	10	+7.5	-15	56	—	+++++	2.7
"	10	4/4	10	+6.4	-27	51	—	+++++	2.3
"	25	4/4	8	+2.2	-58	60	—	—	1.7
2 Aminothiazole	10	39/40	9	+4.4	-27	36	—	+++++	2.5
2 Aminothiazole SO ₄	0.5	4/4	9	+3.4	-12	14	—	+++	1.9
"	10	4/4	9	+3.7	-3	77	—	++++	2.0
"	15	4/4	9	+2.8	-26	80	—	++++	1.8
"	20	4/4	9	+2.8	-26	50	—	++++	2.1
Dithane	10	4/4	9	+3.5	-8	65	—	+++	2.3
"	15	4/4	9	+1.6	-58	71	—	+++++	1.9
"	20	4/4	9	+3.2	-17	27	—	+++	1.8
Bis (4 acetaminophenyl) selen dihydroxide	10	8/8	9	-9	-117	35	—	++++	1.1
Ethylene thiourea	10	4/4	8	+1	-97	33	—	+++	1.5

* The first figure refers to the number of surviving animals, the second to all animals examined
 † Figures refer to number of animals affected

to 0.2%; aminothiazole hydrochloride, 0.025 to 0.05%; acetyl 2-aminothiazole, 0.25%; monoacetyl thiourea, 0.1%; benzalthiobarbituric acid, 0.2%; p,p'-diaminobenzophenone, 0.25%; 2-mercapto-3-phenyl-4,6,6-trimethyl hydropyrimidine, 0.25%; p-phenylenediamine, 0.25%; Dithane, 0.1 to 0.25%; ethylene thiourea, 0.1%; bis-(4-acetaminophenyl) selenium dihydroxide, 0.05 to 0.1%.

The most active compounds were propylthiouracil, thiouracil, 2-aminothiazole, and Dithane. Propylthiouracil was the most effective of these, 0.01% of this drug causing the most severe hyperplasia that was observed in any of the experiments; 0.01% thiouracil caused less than half as much. Dithane was approximately as active as thiouracil, and 2-aminothiazole was less than half as active.

In a few instances degeneration of the thyroid gland in addition to hyperplasia was seen after the administration of selenium compounds, 2-aminothiazole, thiouracil, and propylthiouracil, but was of regular occurrence after the administration of diaminobenzophenone and p-phenylenediamine.

TABLE II

Macrofollicular effect of thiouracil and 2 aminothiazole if administered for a short period only (8-10 days)

		FOLLICULAR SIZE		
		Small	Medium	Large
Control		10	23	0
Thiouracil	.02%	0	2	4
	.05%	0	5	24
2-Aminothiazole	.02%	0	1	0
	.05%	0	2	1
	.1%	0	2	3

Two general effects of the compounds appeared to be particularly significant: failure to gain the normal amount of weight, and the appearance of morphologic changes in the thymus and other lymphoid structures.

Failure to gain the normal amount of weight was common (Table III), and actual loss of weight occurred in rats that had received diacetylthiourea, 0.2%; selenium dioxide, 0.0025 and 0.005%; bis-(4-acetaminophenyl) selenium dihydroxide, 0.1%; sodium selenate, 0.005%; sodium selenite, 0.0025 and 0.005%; 2-mercapto-3-ethanol-4,6,6-trimethyl hydropyrimidine, 0.25%; 2-mercapto-4,6,6-trimethyl hydropyrimidine, 0.25%; 2-mercapto-3-(o-tolyl)-4,6,6-trimethyl hydropyrimidine, 0.25%; p-phenylenediamine, 0.25%; isonicotinic acid, 0.25%.

Of the compounds that were found to be the most effective goitrogenic agents in this study, 0.02 or 0.05% thiouracil in the diet usually caused a gain in weight greater than occurred in the normal controls (Table III), but in some instances the gain in weight was less (3 to 19% less). On the other hand, 0.01 to 0.1% propylthiouracil caused less gain in weight (15 to 27%), as did 0.025 to 0.2% 2-aminothiazole (up to 58%). Dithane affected the normal rate of growth only in concentrations higher than 0.15%, at which level the gain in weight was 8 to 17% less than the controls.

TABLE III

List of compounds of series III showing general disturbances

	DOSAGE	NUMBER OF ANIMALS	CHANGE IN GROWTH RATE % OF NORMAL	THYROID		THYMUS	
				Weight	Hyperplasia	Weight	Change % of normal
	%			mg./ gm.	degree	mg./ gm.	
SG Control		4		.112	0	3.5	± 0
(9) 2-Aminothiazole	.1	4	-57	.414	++++	2.7	-23 Status after karyor- rhesis in spleen
Dithane	.2	4	-17	.272	+++	1.8	-49
"	.15	4	-58	.714	+++++	1.9	-46
"	.1	4	-8	.654	+++	2.3	-34
"	.05	4	± 0	.177	++	2.5	-28
2-Aminothiazole SO ₄	.2	4	-26	.499	++++	2.1	-40
"	.16	4	-26	.802	++++	1.8	-49
"	.1	4	-3	.768	+++	2.0	-43
"	.05	4	-12	.143	+++	1.0	-46
SH Control		4		.131	+	3.4	± 0
(9) 2-Aminothiazole	.1	4	-33	.548	+++++	2.3	-32
Bis-(4-acetaminophenyl) selenium dihydroxide	.1	4	-100	.370	++++	1.2	-65 Marked atrophy of thymus
2-Mercapto-3-ethanol- 4,6,6-trimethyl hy- dro pyrimidine	.25	3	-160	.160	atrophy	.8	-76 Adv. deplet. of thymus; atrophy of sal. gl. and kidneys.
2-Mer.3-(o-tolyl)-4,6,6- trimethyl hydroxyri- midine	.25	4	-105	.218	++	1.6	-53 Considerable atrophy of thymus; atrophy of sal. gl. and kidneys.
SI Control		4		.162		3.1	± 0
(8) 2-Aminothiazole	.1	4	-35	.450		2.1	-32
Propylthiouracil	.25	4	-58	.600		1.7	-45
SJ Control		4		.121	0	2.7	± 0
(8) 2-Aminothiazole	.1	4	+3	.507	++++	2.5	-7
Ethylene thiourea	.1	4	-97	.385	+++	1.5	-44 Marked atrophy of thymus
SK Control		4		.134	0	3.1	± 0
(8) 2-Aminothiazole	.1	4	+17	.486	++++	2.7	-13
Bis-(4-acetaminophenyl) selenium dihydroxide	.1	4	-134	.329	+++++	1.0	-65 Adv. deplet. of thymus
SL Control		4		.124	0	4.1	± 0
(9) 2-Aminothiazole	.1	4	-24	.449	+++++	2.3	-44
SM Control		4		.112	0	2.9	± 0
(11) 2-Aminothiazole	.1	4	-49	.156	+++	2.8	-3
SN Control		4		.12	0	3.3	± 0
(10) 2-Aminothiazole	.1	4	-34	.14	++	2.8	-15

TABLE III—Continued

	DOSAGE	NUMBER OF ANIMALS	CHANGE IN GROWTH RATE % OF NORMAL	THYROID		THYMUS	
				Weight	Hyperplasia	Weight	Change % of normal
	%			mg./gm.	degree	mg./gm.	
SP Control		4		.15		3.6	±0
(10) 2-Aminothiazole	.1	4	-12	.18		2.6	-28
Thiouracil	.05	4	-31	.45		2.5	-31
SQ Control		4		.23	0	3.6	±0
(10) 2-Aminothiazole	.1	3	-42	.28	+++	2.5	-31
Thiouracil	.05	4	-19	.53	+++++	2.8	-22
"	.02	4	-7	.34	+++++	3.25	-10
"	.01	4	-3	.29	+++	3.3	-8
Propylthiouracil	.1	4	-27	.51	+++++	2.3	-36
"	.05	4	-15	.56	+++++	2.7	-25
"	.01	3	-18	.63	+++++	2.9	-19

In summarizing the significant histologic changes it should be pointed out that pyknonecrosis of thymus, lymph nodes and spleen, and salivary glands was seen in isolated instances among both untreated and dosed animals. Consequently, the material sufficed for microscopic study and evaluation only when it was available from several animals exposed to the same drug and showing pathologic changes.

Such material was available for thiouracil; 2-aminothiazole; propylthiouracil; Dithane; *β*-isothiouracil propionic acid; monoacetyl thiourea; diacetyl thiourea; pseudothiohydantoin; 2-thiohydantoin-5-acetic acid; 2-thiohydantoin-5-propionolactam; 2-thiohydantoin-5-propionic acid; 2-methyl-3-nitro-4-ethoxymethyl-5-cyano-6-chloropyridine; thialdine; thiamine; bis-(4-acetaminophenyl) selenium dihydroxide; antipyrine; aminopyrine; selenium dioxide; sodium selenite. Only the three selenium compounds were found to be markedly toxic.

Advanced pyknonecrosis of thymus, lymph nodes and Malpighian bodies of spleen, and marked pyknonecrosis of salivary glands and adrenals occurred in animals that died following the administration of bis-(4-acetaminophenyl) selenium dihydroxide (0.1%), selenium dioxide or sodium selenite (0.005%), necrosis of liver and kidneys occurred in several, and marked degeneration of the thyroid gland in some. Advanced lymphocytic depletion of the thymus and some atrophy of the lymphatic tissues occurred in four animals that had received 0.1% bis-(4-acetaminophenyl) selenium dihydroxide with 0.5% p-amino-benzoic acid, and in some after 0.0025% selenium dioxide or sodium selenite, but the salivary glands and thyroid appeared to be essentially normal. No toxic changes were found in four animals that had received 0.05% bis-(4-acetaminophenyl) selenium dihydroxide for 9 days.

It is of interest to note that the inorganic selenium compounds were non-goitrogenic, and intensely toxic when fed in very small amounts. On the other hand, the aromatic bis-(4-acetaminophenyl) selenium dihydroxide was ap-

parently innocuous at these same concentrations, but was both goitrogenic and toxic when fed in much larger amounts. The action was more in line with the sulfur and carbon analogs than with the selenium content. Aromatic selenium compounds not containing the amino group, like their carbon and sulfur analogs, were neither goitrogenic nor toxic when fed in relatively large amounts.

Monoacetyl thiourea or diacetyl thiourea (0.05 or 0.1%) and aminopyrine (1.0%) caused atrophy of the thymus in many of the animals, but there was no significant alteration of the thyroid gland or other tissues.

When 2-aminothiazole was administered, no toxic changes were observed generally even when 0.2% was given. The thyroid revealed occasional degenerative changes, but these differed in no respect from those observed with thiouracil.

The following compounds, for which only one animal was available for microscopic study, caused some thymus atrophy: 3-carboxy-2-pyridone, 0.2%; 2,4-diketo-1,2,3,4-tetrahydro-5,6-(2',3'-pyrido) pyrimidine, 0.2%; 3-cyano-4-carbethoxy-5-nitro-6-methyl-2-pyridone, 0.2%; 3-cyano-4-ethoxymethyl-6-methyl-2-pyridone, 0.2%; acetyl 2-aminothiazole, 0.25%; diaminobenzophenone, 0.25%; 2-mercapto 3-ethanol-4,6,6-trimethyl hydropyrimidine, 0.25%; p-phenylenediamine, 0.25%; dimethylaminoazobenzene, 0.25%. Marked lymphocytic depletion of thymus and lymph nodes was caused by mixed ethyl and dimethyl mercaptothiazole; isonicotinic acid, 0.25%; arsanilic acid, 0.25%. Of these compounds, only acetyl 2-aminothiazole, diaminobenzophenone, and p-phenylene diamine were goitrogenic. Only 2-mercapto-3-ethanol-4,6,6-trimethyl hydropyrimidine, p-phenylenediamine, and dimethylaminoazobenzene caused considerable degeneration of the thyroid gland.

These striking changes in the thymus were paralleled by a marked decrease in its weight (Table I, Series III). The thymus of normal control animals weighed 2.7 to 4.1 mgm./gram of body weight (average 3.3 mgm./gram), but in only 2 out of 10 groups was it less than 3.1 mgm./gram, and in 1 more than 3.6 mgm./gram. It is noteworthy that only 11 of 40 experimental groups had thymuses that weighed more than 2.7 mgm./gram; the others weighed considerably less. The decrease in weight of the thymus occurred simultaneously with the decrease in rate of growth of the animal, and frequently was conspicuous when the animal was still growing at a normal rate. The thymus, therefore, is a very sensitive indicator of general body disturbances produced by many types of chemical compounds.

Only reductions in thymus weight of more than 50% could be recognized microscopically. When the changes were of this severity, a good correlation was found between body weight, thymus weight, and the microscopic appearance of the thymus (Table IV).

Using the weight of the thymus as an indicator, it was found that selenium and mercapto compounds were the most effective in provoking general disturbances (Table III). The most goitrogenic compounds were also capable of provoking these changes. If the comparison is based on equivalent goitrogenic concentrations, differences are not marked: 0.01% propylthiouracil caused a thymus weight loss of 19%; 0.05% thiouracil, 26% 0.1% 2-aminothiazole, 23%; 0.05% Dithane, 28%. Therefore, when equivalent goitrogenic doses were ad-

ministered to rats, there did not appear to be much difference in the toxicity of these compounds (Table V).

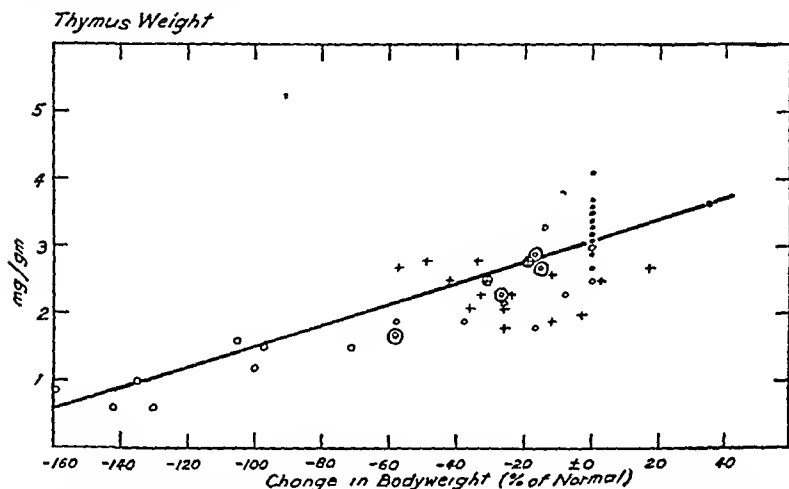


FIG 4 WEIGHT OF THYMUS (MG/M PER GRAM OF BODY WEIGHT) AS COMPARED WITH CHANGE IN BODY WEIGHT (PER CENT OF NORMAL)

● normal controls, ⊕ thiouracil controls, + 2 aminothiazole controls; ⊙ propyl thiouracil controls, ○ other compounds tested

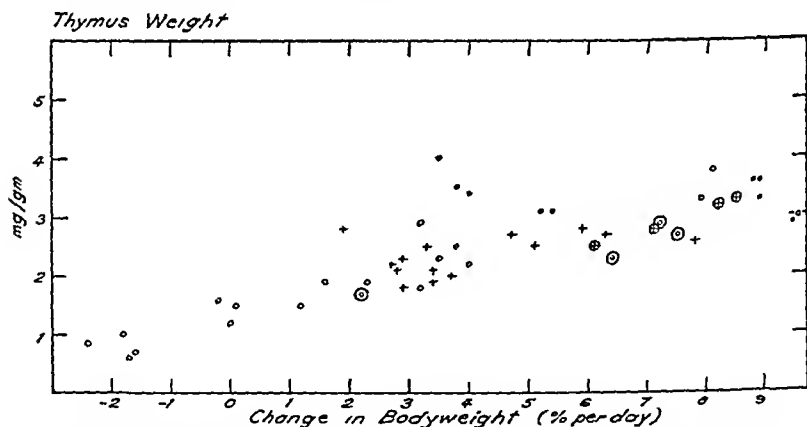


FIG 5 WEIGHT OF THYMUS (MG/M PER GRAM OF BODY WEIGHT) AS COMPARED WITH CHANGE IN BODY WEIGHT (PER CENT PER DAY)

● normal controls, ⊕ thiouracil controls, + 2 aminothiazole controls, ⊙ propyl thiouracil controls, ○ other compounds tested

DISCUSSION. The hyperplasia of the thyroid gland was undoubtedly due to the specific action of the compounds that were administered. Whether the

general changes, particularly the weight loss of the thymus, were produced by the compounds directly or by refusal to eat the adulterated diet was studied

TABLE IV

Correlation between body weight, thymus weight and microscopic appearance of thymus of all rats that showed microscopic evidence of wasting of thymus

REDUCTION IN GAIN OF BODY WEIGHT (% OF NORMAL)	REDUCTION IN THYMUS WEIGHT (% OF NORMAL)	MICROSCOPIC APPEARANCE OF THYMUS
40-70	34	Some atrophy
70-110	55	Considerable to marked atrophy
110-230	77	Advanced depletion of lymphocytes

TABLE V

Goitrogenic effect as compared with change in body weight and weight of thymus

MATERIAL		NUMBER OF RATS*	DURA- TION OF EX- PERI- MENT	THYROID		% CHANGE IN BODY WEIGHT		THYMUS	
				Average weight	Hyperplasia	Per day	Of normal	Average weight	Weight change % of normal
%		days	mg./ gm.	degree			mg./ gm.		
Normal controls		40/40	9	.14	0	+6.1	±0	3.3	±0
Thiouracil	.01	4/4	10	.29	+++	+8.5	-3	3.3	-8
"	.02	4/4	10	.34	+++++	+8.2	-7	3.25	-10
"	.05	8/8	10	.50	+++++	+6.6	-25	2.8	-26
Propylthiouracil	.01	3/4	10	.63	+++++	+7.2	-18	2.9	-19
"	.05	4/4	10	.56	+++++	+7.5	-15	2.7	-25
"	.10	4/4	10	.51	+++++	+6.4	-27	2.3	-36
"	.25	4/4	8	.60	—	+2.2	-58	1.7	-45
2-Aminothiazole SO ₄	.05	4/4	9	.14	+++	+3.4	-12	1.9	-46
"	.10	4/4	9	.77	+++	+3.7	-3	2.0	-43
"	.15	4/4	9	.80	++++	+2.8	-26	1.8	-49
"	.20	4/4	9	.50	++++	+2.8	-26	2.1	-40
2-Aminothiazole	.10	39/40	9	.36	+++++	+4.4	-27	2.5	-23
Dithane	.05	4/4	9	.18	+++	+3.8	±0	2.5	-28
"	.10	4/4	9	.65	+++++	+3.5	-8	2.3	-34
"	.15	4/4	9	.71	+++++	+1.6	-58	1.9	-46
"	.20	4/4	9	.27	+++	+3.2	-17	1.8	-49

* See footnote, Table I.

by a starvation experiment. The daily intake of food of 6 male and 6 female rats, each with a body weight of approximately 75 grams, was restricted to 25% of average. Five animals died on the third day after having lost 23% of the

body weight and 69% of the thymus weight. Changes were somewhat more severe in the four animals that died on the fourth day when the loss of body weight was 25% and the thymus weight loss was 78%. The adrenals of all these animals had gained weight. All the animals were dead on the sixth day. Advanced pyknonecrosis of the lymphocytes of the thymus, and phagocytosis of nuclear debris by macrophages was seen on microscopic examination; the adrenals revealed advanced depletion of fat from the zona fasciculata and retention of fat in the zona glomerulosa.

Starvation thus resulted in both the gross and microscopic picture of the alarm reaction, confirming the contention of Selye (5, 1) that even fasting must be regarded as an alarming stimulus. These findings clearly indicate that the above mentioned general changes are to be regarded as phenomena of the general adaptation syndrome, but they do not differentiate whether this was brought about by the toxic agent itself or by starvation.

SUMMARY

1. Seventy-eight compounds were screened for goitrogenic action. Of these, 12 were found to be effective, but only thiouracil, propylthiouracil, 2-aminothiazole and Dithane were found to be markedly active.

2. Of the four markedly goitrogenic compounds, propylthiouracil was the most effective, followed by thiouracil and Dithane. 2-Aminothiazole seemed to be less than half as active as thiouracil.

3. The weight of the thyroid gland, its iodine content and its microscopic appearance were all found to be equally useful criteria for goitrogenic action.

4. Many compounds caused general disturbances such as retardation or arrest in growth of the animals, atrophy or necrosis of the thymus and other lymphatic structures, and degeneration of various excretory and endocrine glands. It appears that these were phenomena of the general adaptation syndrome of Selye.

5. Evidence has been presented to show that the thymus is a most sensitive indicator of general disturbances of this kind, its weight being an excellent measure of the alarm reaction.

6. Using the weight of the thymus as an indicator of general disturbances, it was found that not much difference seemed to exist in the toxicity of the four most effective goitrogenic compounds administered in equally effective doses.

We are indebted to Dr. W. F. Bruce and Mr. Lester Szabo of these laboratories and to the B. F. Goodrich Co. and the Rohm and Haas Co. for the compounds used in this study.

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A STUDY OF THE EFFECTS OF 1-AMINO-1-PHTHALIDYL-PROPANE HYDROCHLORIDE ON THE EXCISED AND INTACT INTESTINE AND UTERUS¹

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Only one report touching on the effects of 1-amino-1-phthalidylpropane hydrochloride, a promising analgesic drug, on the intestine has appeared in the literature (1). Because of this meager information on the action of this compound on the intestine and because there is no information available on its effects on the uterus, this investigation was undertaken.

METHODS *Excised intestine* Excised longitudinal segments of rabbit intestine were used. The methods of experimentation employed were the same as those previously described by us (2).

The drugs were weighed and dissolved in Tyrode's solution just before each experiment. Adjustments were made to maintain a constant pH. A given amount of this solution was added to the bath to make the necessary dilutions. After exposure of the intestine for approximately 3 to 4 minutes to the Tyrode's solution containing the drug, the solution was drained off and fresh Tyrode's solution added to the cylinder. Both racemates A and B (3) and aminopyrine were used in dilutions of M/5,000, M/2,000, M/1,000, and M/500, and occasionally in lower and higher concentrations.

Intact intestine For these experiments unanesthetized dogs with thirty Vella loops of the ileum, the jejunum or the duodenum were used. Except for the fact that an end to end anastomosis of the gut was made in re-establishing its continuity, the method of preparation of the loops and the method of recording the contractions and changes in the general tonus of the intestine were the same as those previously described by one of us (4). The pressure in the balloon within the gut was either 15 or 20 cm. water pressure. The racemates were injected intravenously in doses of 25, 30, 40 and 50 mg./kg.

Excised uterus Experiments similar to those on the excised intestine were also performed on the excised rabbit uterus using the same equipment. A segment was taken from each horn of the uterus and the two segments were studied simultaneously. Locke's solution was used in place of Tyrode's solution as the bath and the drugs were dissolved in this solution instead of in Tyrode's solution. Both racemates A and B were used in final dilutions of M/5,000, M/2,000, M/1,000, M/500 and M/250. The bath was kept at pH 7.6 and at a temperature of $37.5^{\circ}\text{C} \pm 0.2^{\circ}$.

Intact uterus Rabbits, cats and dogs were used in studying the action of 1-amino-1-phthalidylpropane hydrochloride on the intact uterus. The method described by Barbour (5) with slight modifications was used in recording the changes in uterine activity. Either ether followed by cycloheptenylethyl barbiturate, or urethane were used as the anesthetics in both cats and rabbits and ether alone in the dogs. The drugs were injected intravenously in doses of 25, 50 and 100 mg./kg. doses.

To rule out anoxia due to cessation of respiration caused by the drug (6), as a possible cause of any change in the activity of the intact uterus, artificial respiration was started before the injections were made. To check the response of the uterus posterior pituitary was injected intravenously at varying times in doses of 1 to 5 U. S. P. units.

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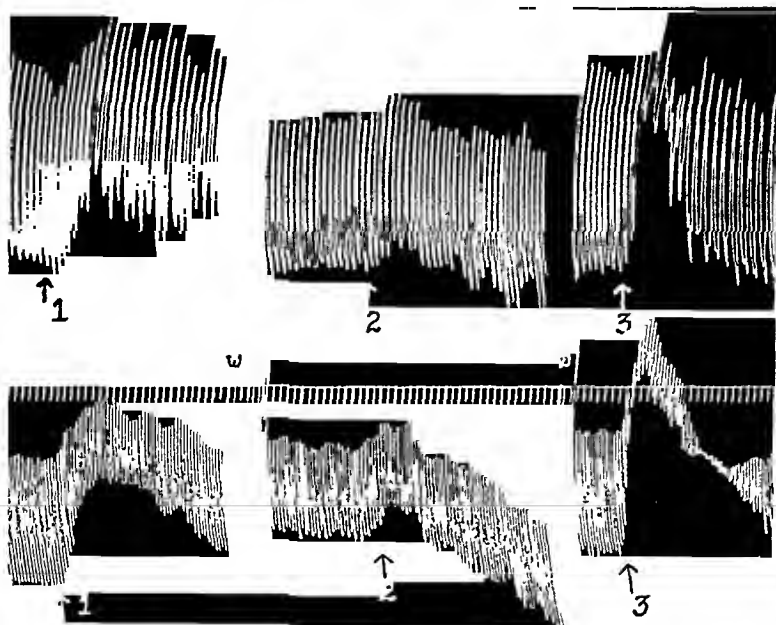


FIG. 1. EXCISED SEGMENTS OF RABBIT INTESTINE

Top record is that of the ileum and the bottom curve that of the duodenum. Up stroke of the lever indicates contraction and the down stroke relaxation. The time is recorded in intervals of 10 seconds.

1. Racemate B of 1-amino-1-phthalidylpropane hydrochloride M/1,000.

2. Racemate A of 1-amino-1-phthalidylpropane hydrochloride M/2,000.

3. Racemate B M/500.

W. Washed specimens and replaced the drug—Tyrode's solution with fresh Tyrode's solution.

TABLE I

RACEMATE	NUMBER OF EXPERIMENTS	M/5,000		M/2,000		M/1,000			M/500		
		+	-	+	-	+	+-	-	+	+-	-
Results on freshly excised intestinal segments											
A	46		3	2	5		5	17		3	11
B	24			1		2	-5	3	1	9	3
Results on intestine kept in a refrigerator for 24 hours											
A	24	1	3		4			10			6
B	24		1		3			11		3	6

+ indicates contraction; +- indicates contraction followed by relaxation; - indicates relaxation.

RESULTS. *Excised intestine.* Seventy experiments were performed with racemate A and 48 experiments with racemate B and all were done on 36 seg-

ments of intestine taken from 8 rabbits. Seventy experiments were done on freshly excised segments of intestine, and 48 similar experiments on segments

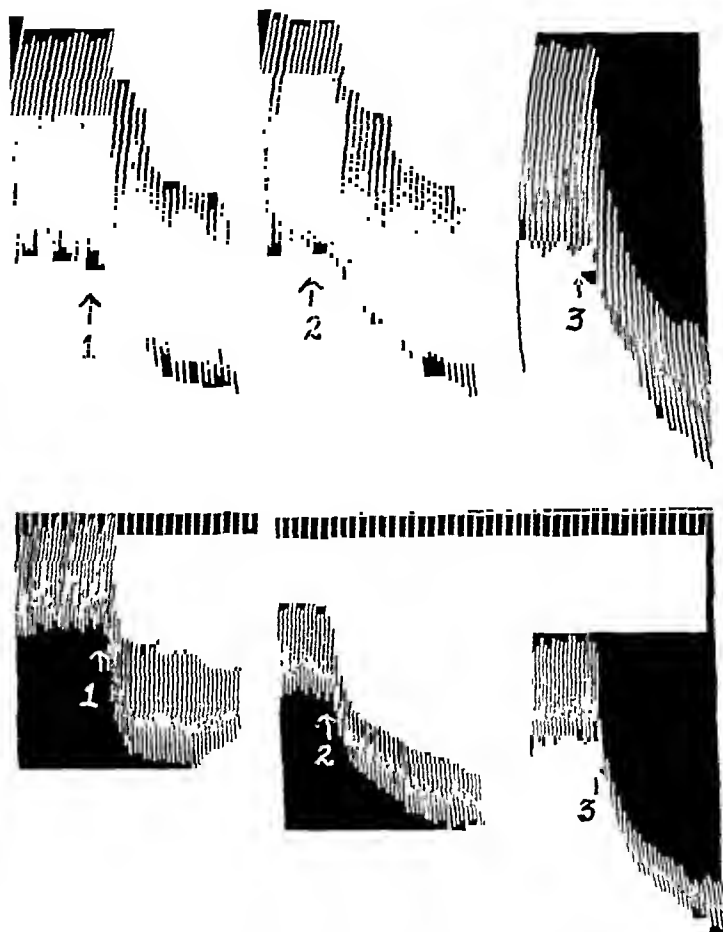


FIG. 2. EXCISED SEGMENTS OF RABBIT INTESTINE

Top record is that of the jejunum and bottom record that of the duodenum. Before this record was made the intestine was stored in a refrigerator in Locke's solution for about 18 hours. Up stroke in the record of intestine indicates contraction. Time is in intervals of 10 seconds.

1. Racemate B of 1-amino-1-phthalidylpropane hydrochloride M/500.
2. Racemate A, M/1,000.
3. Racemate A, M/500.

which had been kept in a refrigerator for 12 to 24 hours. In twenty-eight (40 per cent) of the former experiments, either an increase or an increase followed by a decrease in the general tonus of the segments was noted (see figure 1).

In the 48 latter experiments an increase in the general tonus was encountered only once and in two instances an increase followed by a decrease was observed (see table I and figure 2).

A few experiments were also performed on excised segments of intestine in which either histamine acid phosphate, acetylcholine chloride or pilocarpine

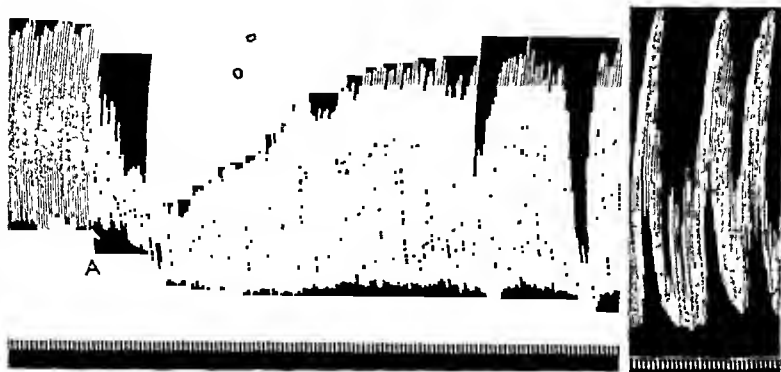


FIG. 3. DOG, 16 KG., WITH THIRY-VELLA LOOP OF THE JEJUNUM

Up stroke of lever indicates contraction and down stroke relaxation of the gut. Time in intervals of 10 seconds. At ↑, 30 mg./kg. of racemate A of 1-amino-1-phthalidylpropane hydrochloride were injected intravenously. The added curve was taken 10 minutes later.



FIG. 4. DOG, 22 KG, WITH THIRY-VELLA LOOP OF JEJUNUM

Up stroke of lever indicates contraction. Time in intervals of 10 seconds. The immediate upswing of the recording lever, at 1, is due to the contraction of the abdominal muscles.

1. Racemate B of 1-amino-1-phthalidylpropane hydrochloride 50 mg./kg injected intravenously.

2. Thiopental sodium (Pentothal sodium), 5 mg./kg. injected intravenously.

nitrate was added to the bath before the addition of 1-amino-1-phthalidylpropane hydrochloride. In these experiments definite antagonism was noted.

Aminopyrine in all concentrations used, caused a prompt decrease in the height of the rhythmic contractions of the intestine and at the same time a decrease in the general tonus.

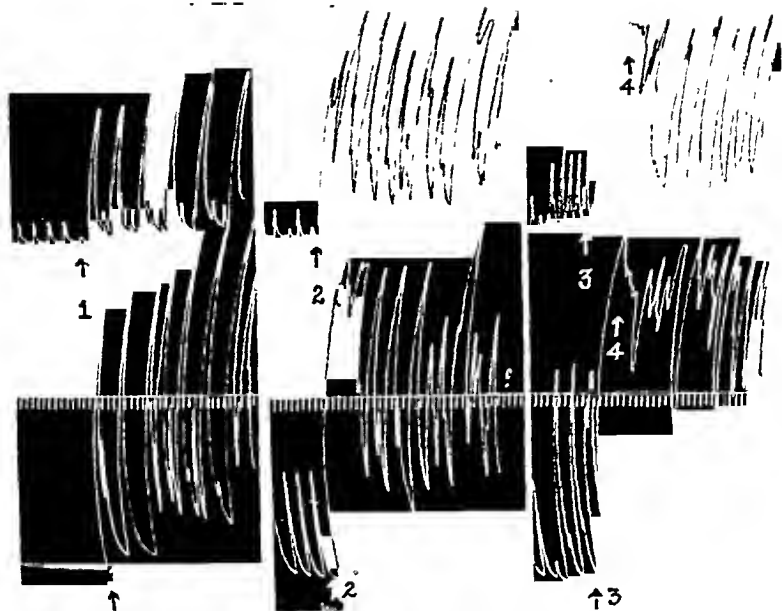


FIG. 5. EXCISED SEGMENTS OF RABBIT UTERUS

Up stroke in curve indicates contraction. Locke's solution used as the bath. Time in intervals of 10 seconds.

1. Racemate B, M/500 solution.
2. Racemate A, M/500 solution.
3. Posterior pituitary, 1 U. S. P. unit added to 100 cc. solution.
4. Racemate A, M/500 solution.

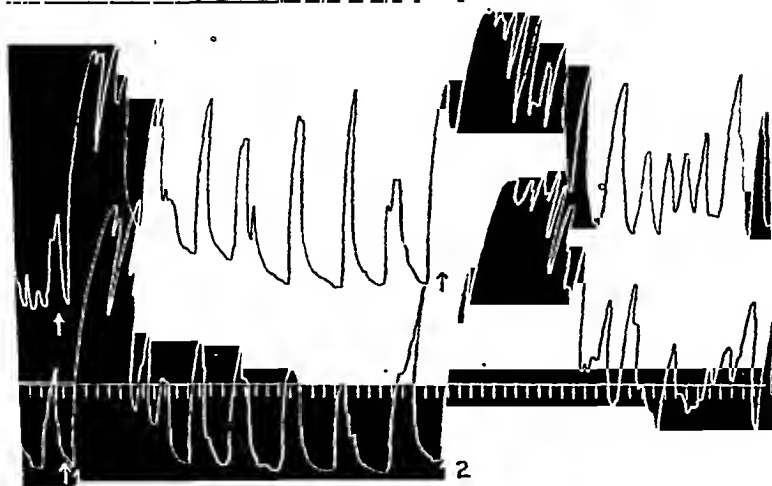


FIG. 6. INTACT UTERUS OF RABBIT UNDER CYCLOHEPTENYLETHYL BARBITURATE

ANESTHESIA AND ARTIFICIAL RESPIRATION

Time in intervals of 20 seconds. Up stroke in record indicates contraction of the uterus.

1. Racemate B, 50 mgm./kgm. injected intravenously.
2. Posterior pituitary, 2 U. S. P. units injected intravenously.

Intact intestine. A total of 26 experiments were performed upon 6 dogs with Thiry-Vella loops. In one animal (see figure 3) 8 separate injections of varying

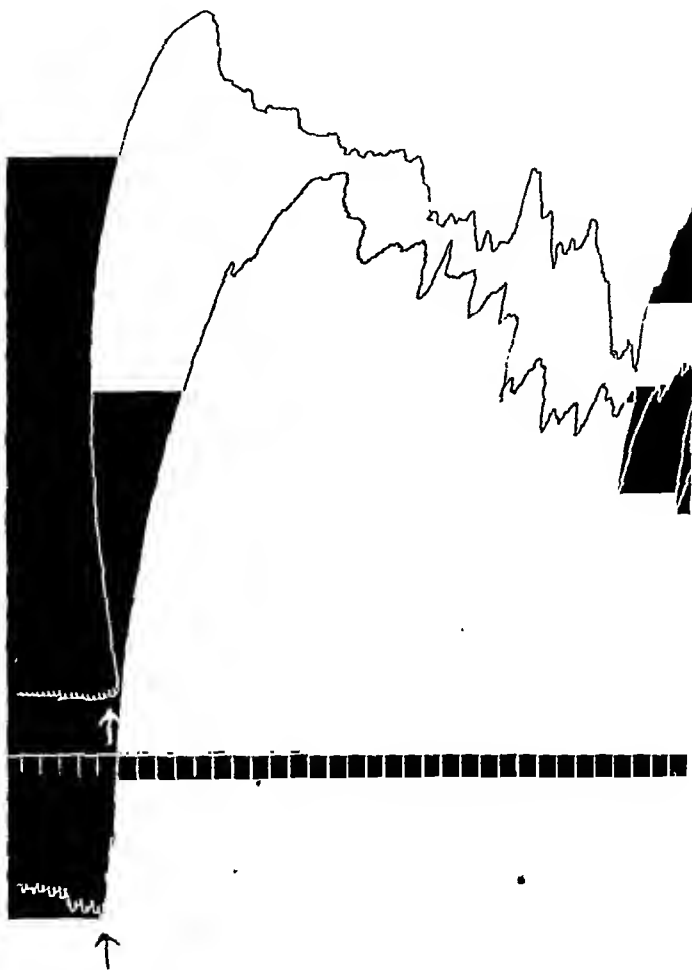


FIG. 7. INTACT UTERUS OF 13 KG. DOG UNDER ETHER ANESTHESIA AND ARTIFICIAL RESPIRATION

Up stroke of lever indicates contraction of uterus. At the \uparrow 100 mg./kg. of racemate B were injected intravenously.

doses of 1-amino-1-phthalidylpropane hydrochloride were made. In each instance a decrease in the height of the rhythmic contractions and a prolonged

decrease in the general tonus of the gut resulted. In the remaining five animals in which 18 similar injections were made a prolonged increase in the general tonus of the gut was observed (see figure 4). The animal used in figure 3 gave peculiar responses to other drugs e.g. morphine sulfate given intravenously caused relaxation of the intestine instead of the usual contraction. In the five dogs in which 1-amino-1-phthalidylpropane hydrochloride increased the general tonus of the gut hexobarbital soluble, vinbarbital sodium, thiopental sodium and thioethamyl sodium when given intravenously antagonized the effect (see figure 4 at 2).

Excised uterus. Fifty-nine experiments were performed in which eleven pairs of excised uterine segments, taken from 8 rabbits, were used. In some instances the drug appeared to initiate rhythmic contractions in segments previously not active as can be seen in figure 5 at 1. In all but 3 experiments a definite increase in the general tonus and in the rate and force of the rhythmical contractions of the uterine segments were produced by the addition of the drug to the bath. As can be seen in figure 5, at 4, 1-amino-1-phthalidylpropane hydrochloride had little if any further effect on uterine segments which had already been contracted by the addition of 1 U. S. P. unit of posterior pituitary added to the bath. Nor did it have any effect upon uterine segments which had been contracted by the addition of either acetylcholine chloride or of histamine acid phosphate to the bath.

Intact uterus. Racemates A and B of 1-amino-1-phthalidylpropane hydrochloride were injected intravenously twenty-two times in 9 cats, and in these contraction of the uterus was seen in fifteen instances and relaxation in seven. Fourteen similar injections were made in 6 rabbits and in these contraction of the uterus occurred ten times (see figure 6) and relaxation in four experiments. Six injections were made in two dogs and in these only an increase in the general tonus of the intact uterus was observed (figure 7).

SUMMARY AND CONCLUSIONS

1. Segments of excised rabbit intestine exposed to 1-amino-1-phthalidylpropane hydrochloride usually respond by a decrease in general tonus. The state of the tissue at the time of exposure to the drug seems to determine the results. If the tissues are in a contracted state relaxation usually results, if relaxed increased general tonus occurs.

2. On the excised rabbit intestine 1-amino-1-phthalidylpropane hydrochloride has an action antagonistic to histamine acid phosphate, to acetylcholine chloride and to pilocarpine nitrate.

3. Aminopyrine in the concentrations used decreases the general tonus of excised segments of rabbit intestine.

4. In five of the six dogs studied 1-amino-1-phthalidylpropane hydrochloride caused an increase in the general tone of the intact intestine. Barbiturates and thiobarbiturates can antagonize the above effect.

5. Excised uterine segments respond to 1-amino-1-phthalidylpropane hydrochloride by a sudden increase in general tonus. In some instances, when the

drug was added to the bath, rhythmic contractions were started in otherwise inactive segments.

6. On uterine segments no antagonism between 1-amino-1-phthalidylpropane hydrochloride and histamine, acetylcholine or posterior pituitary was observed.

7. The predominant effect of 1-amino-1-phthalidylpropane hydrochloride on the intact uterus of rabbits, cats and dogs is that of stimulation.

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STUDIES WITH TETRAZOLE DERIVATIVES

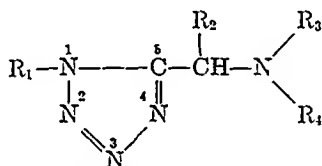
IV. SOME PHARMACOLOGIC PROPERTIES OF AMINOMETHYL TETRAZOLES¹

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In the first three papers of this series (1-3), a group of pentamethylene tetrazole derivatives, a group of 1,5-disubstituted tetrazole derivatives, and a group of 5-amino tetrazole derivatives were studied in an effort to determine their convulsant and analeptic actions as compared with pentamethylene tetrazole. The presence of the amino group in the 5 position of the tetrazole ring caused a decrease in the analeptic activity of the tetrazole nucleus, but these compounds generally were highly convulsant. The fourth group of tetrazole derivatives, with which the present report is concerned, consists of 38 derivatives of tetrazole in which a variety of alkyl or substituted phenyl groups has been introduced in position 1 and a series of mono-, di-, and trisubstituted aminomethyl groups in position 5. These compounds differ from those of the third group in that a carbon atom has been placed between the amino group and the tetrazole ring.



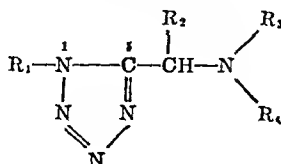
The structures of the substituted aminomethyl tetrazoles were varied systematically and an attempt was made to correlate the changes in chemical structure and pharmacologic activity. The outline of systematic alterations of substituent groups is presented in table 1, along with comments on the general and specific actions of the 38 compounds tested. None of these compounds to our knowledge has been previously described.

EXPERIMENTAL. The action of these compounds upon the central nervous system was studied by the injection intraperitoneally of graded doses into albino rats of approximately equal weights. Convulsive, sedative, and lethal effects were recorded. The arousal action of the drugs during pentobarbital-induced anesthesia in rats and rabbits was also studied in some cases. The criteria of arousal were identical with those listed in previous papers of this series. Three stages of sedation were roughly outlined: (1) mild sedation—lack of most spontaneous movement, (2) heavy sedation—ability to assume upright position, reflexes present, (3) anesthesia. In one case, rabbits were given the compound intravenously to check the sedative action in another species.

DISCUSSION. The insertion of a methylene group between the carbon of the tetrazole nucleus and the amino group, as in this series, brought about a further

¹ These compounds were prepared by E. Bilhuber, Inc., Orange, New Jersey.

TABLE 1
*Structure and pharmacologic actions**



COMPOUND NUMBER	R ₁	GENERAL ACTION	COMMENTS
$R_2 = H; R_3 = R_4 = C_6H_5$			
TT-167	CH ₃	Mild sedation and convulsions	500 mg./kg.; intermittent convulsions and sedation. No arousal in nembutalized rats.
TT-172	C ₆ H ₅ CH ₃	Mild sedation and convulsions	100-200 mg./kg., sedation; 200-500, sedation followed by convulsions; 500, fatal.
TT-173	p-CH ₃ C ₆ H ₄	Heavy sedation and convulsions	100-300 mg./kg., sedation, no anesthesia; 750-1000, sedation with intermittent convulsions.
TT-147	p-CH ₃ OC ₆ H ₄	Mild sedation and convulsions	100-250 mg./kg., intermittent sedation and convulsions; 250, fatal.
TT-169	p-OHC ₆ H ₄	Mild sedation and convulsions	200-500 mg./kg., sedation with intermittent convulsions; 500, convulsions and death.
TT-162	p-NO ₂ C ₆ H ₄	Convulsions	100-200 mg./kg., convulsions, not fatal at 500; 1000, no arousal in nembutalized rats
TT-150	m-NO ₂ C ₆ H ₄	Heavy sedation and convulsions	50-150 mg./kg., sedation; 750-1000, sedation, muscular tremors and death.
TT-162	m-NH ₂ C ₆ H ₄	Mild sedation and convulsions	50-200 mg./kg., sedation, 200 up, intermittent convulsions and sedation; 800, fatal.
TT-170	β -C ₁₀ H ₇ β -naphthyl	Anesthesia	100 mg./kg., heavy sedation; 200, anesthesia for 1 hour; 900, fatal. Not effective orally. Rabbits IV: 10, 15, 20 mg./kg., good anesthesia for 10-15 minutes.
TT-171	o-C ₆ H ₅ -C ₆ H ₄	Mild sedation and convulsions	100-250 mg./kg., sedation; 300, convulsions; 500, fatal.
TT-175	p-C ₆ H ₅ -C ₆ H ₄	Mild sedation	100-500 mg./kg., mild sedation, no anesthesia; larger doses not tried.

TABLE 1—Continued

COMPOUND NUMBER	R ₁		GENERAL ACTION	COMMENTS
	$ \begin{array}{c} \text{CH}_2\text{—CH}_2 \\ \diagup \quad \diagdown \\ \text{R}_2 = \text{H}; \text{R}_3 \text{ and } \text{R}_4 = \text{C} \\ \diagdown \quad \diagup \\ \text{CH}_2\text{—CH}_2 \end{array} $ <p>CH₂, piperidino</p>			
TT-127	i-C ₆ H ₁₁		Convulsions	100 mg./kg., convulsions; 800, fatal.
TT-177	p-C ₆ H ₄ —C ₆ H ₅		Mild sedation	100–500 mg./kg., sedation; no anesthesia. Higher doses not tried.
	R ₁	R ₂		
R ₁ = cycloC ₆ H ₁₁ ; R ₂ = H				
TT-135	H	H	Mild sedation	700–1000 mg./kg., sedation
TT-146	H	n-C ₇ H ₇	Mild sedation and convulsions	50–250 mg./kg., sedation; 250–400, intermittent convulsions; 400, fatal.
TT-144	H	C ₆ H ₅	Mild sedation and convulsions	50–250 mg./kg., sedation; 500, intermittent tremors and sedation; 750, convulsions and fatal.
TT-131	H	cycloC ₆ H ₁₁	Mild sedation	100–800 mg./kg., sedation.
TT-131 Succinamide	$ \begin{array}{c} \text{CO—CH}_2 \\ \\ \text{CH}_2\text{COOH} \end{array} $	cycloC ₆ H ₁₁	No action	1000 mg./kg., no symptoms.
TT-136	CH ₃	CH ₃	Convulsions	150–250 mg./kg., persistent convulsions, recovery up to 600 mg./kg. (highest dose tried).
TT-125	$ \begin{array}{c} \text{CH}_2\text{—CH}_2 \\ \diagup \quad \diagdown \\ \text{C} \\ \diagdown \quad \diagup \\ \text{CH}_2\text{—CH}_2 \end{array} $ <p>piperidino</p>		Heavy sedation and convulsions	200–400 mg./kg., sedation and light anesthesia; 400, up, muscular tremors and latent death.
TT-143	CH ₃	$ \begin{array}{c} \text{CH}_3 \\ \\ \text{—CH} \\ \quad \\ (\text{CH}_2)_2\text{—C—}(\text{CH}_2)_2 \\ \\ \text{OH} \end{array} $	Convulsions	60–80 mg./kg., prompt convulsions; 120, death. No arousal in nembutalized rats.
R ₁ = C ₆ H ₅ ; R ₂ = H				
TT-168	H	CH ₃	Heavy sedation and convulsions	100 mg./kg., sedation; 200, anesthesia; 1000 convulsions and death.
TT-165	H	C ₆ H ₅	Mild sedation and convulsions	50–300 mg./kg., sedation; 300 up, sedation followed by convulsions, death.

TABLE 1—Continued

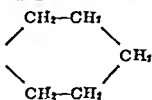
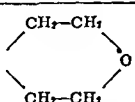
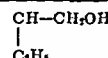
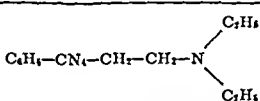
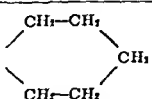
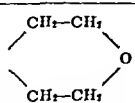
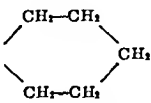
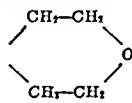
COMPOUND NUMBER	R ₁	R ₂	GENERAL ACTION	COMMENTS
R ₁ = C ₆ H ₅ R ₂ = H—Continued				
TT-145	H	C ₂ H ₅	Mild sedation and convulsions	100-750 mg /kg , sedation, 1000 sedation, tremors, late convulsions, death
TT 153	H	C ₂ H ₅	Mild sedation and convulsions	50-500 mg /kg , sedation 500-600, convulsions 600, death
TT 16(116)	C ₂ H ₅	C ₂ H ₅	Mild sedation and convulsions	30-100 mg /kg , sedation and tremors, 250-300, heavy sedation and tremors
TT-108	 piperidino		Heavy sedation and convulsions	100-400 mg /kg , sedation, 500 up, sedation with intermittent convulsions
TT-104	 morpholino		Heavy sedation and convulsions	100-600 mg /kg , sedation, 600-1000, intermittent convulsions and sedation, 1000, death
TT 155	H	CH ₂ CH ₂ OH	Mild sedation	1000 mg /kg , sedation
TT 159	H		Mild sedation	500-1000 mg /kg , sedation
TT 161			Mild sedation and convulsions	400-750 mg /kg , intermittent convulsions and sedation, 750, fatal
R ₁ = C ₆ H ₅ , R ₂ = CH ₃				
TT-166	H	CH ₃	Heavy sedation	50-100 mg /kg , mild sedation, 100-1000, heavy sedation, no anesthesia
TT-111	C ₂ H ₅	C ₂ H ₅	Mild sedation and convulsions	100-300 mg /kg , sedation, 300-600, intermittent convulsions and sedation, 600, death
TT-114	 piperidino		Heavy sedation	100-800 mg /kg , sedation no anesthesia
TT 118	 morpholino		Mild sedation and convulsions	250 mg /kg , sedation 500 convulsions, 500-800, no arousal in nembutalized rats

TABLE 1—Continued

COMPOUND NUMBER	R ₂	R ₁	GENERAL ACTION	COMMENTS
R ₁ = C ₁₀ H ₇ (naphthyl); R ₂ = H				
TT-164	CH ₃	CH ₃	Heavy sedation and convulsions	100 mg./kg., heavy sedation; 200, anesthesia with slight muscular tremors; 1000, death.
TT-142	C ₂ H ₅	C ₂ H ₅	Anesthesia	50-100 mg./kg., heavy sedation; 200-900, anesthesia; 900, death.
TT-179	 piperidino		Heavy sedation	300-1000 mg./kg., sedation, no anesthesia.
TT-178	 morpholino		Mild sedation and convulsions	100-300 mg./kg., early sedation followed by convulsions; 300-500, convulsions; 800, fatal.

* All compounds were prepared as hydrochlorides except TT-131 Succinamide (Na salt), TT-131 (Base), TT-114 (Bitartrate), TT-150 (Nitrate), TT-175 (Tartrate), and TT-177 (Phosphate).

decrease in the stimulant activities of most of these compounds as compared to those of previous groups. At the same time hypnotic and anesthetic actions became more evident. In fact, a number of the compounds produced depression with no noticeable stimulant action within the dose ranges studied. These are indicated in detail in table 1 and more graphically in table 2.

Two key points of substitution appear to influence greatly the pharmacologic characteristics of the compounds in this group. If no hydrogen is attached to the amino nitrogen, the simultaneous presence in the R₁ position of an isoamyl, cyclohexyl, or p-nitrophenyl radical generally produces a compound with stimulant action only. However, if the R₁ group is a naphthyl or p-diphenyl radical, the resultant compound has only depressant actions. Furthermore, the presence of a phenyl or other mono-substituted phenyl radical in the R₁ post produces a compound with mixed activity, depending upon the dosage. Special attention should perhaps be called to those compounds containing naphthyl radicals. In each instance the depressant action was predominant.

A second type of compound having only depressant action appears to depend largely upon the attachment to the amino nitrogen of two hydrogens or a hydrogen and primary alcohol group. Either phenyl or cyclohexyl groups will serve in the R₁ position. A third type of compound having only depressant action has a methyl radical replacing one of the carbon-bound hydrogens (R₂) of the aminomethyl group. The presence of a hydrogen on the amino group does not seem to be necessary in this type of compound.

The bulk of the compounds, which have both stimulant and depressant actions, are influenced less by the substituents in the R_3 and R_4 positions than by the nature of the R_1 group. Those compounds having mono-substituted phenyl groups in this spot tend to show greater stimulant action and less depressant action than those having merely a phenyl group.

TABLE 2

TYPES OF COMPOUNDS	TYPE FORMULA $ \begin{array}{c} R_3 \\ \diagup \\ R_1-CN-CH-N \\ \diagdown \quad \\ R_2 \quad R_4 \end{array} $
Stimulant only	$ \begin{array}{c} R_1 \\ \hline \left. \begin{array}{l} \text{isoamyl} \\ \text{cyclohexyl} \\ \text{p-nitrophenyl} \end{array} \right\} -CN-CH_2-N \begin{array}{l} \diagup R_3 \\ \diagdown R_4 \end{array} \end{array} $
Both stimulant and depressant	$ \begin{array}{c} R_1 \\ \hline R-C_6H_4-CN-CH_2-N \begin{array}{l} \left\{ \begin{array}{l} H, R_4 \\ R_3, R_4 \end{array} \right. \\ \downarrow \\ C_6H_5-CN-CH_2-N \begin{array}{l} \left\{ \begin{array}{l} H, R_4 \\ R_3, R_4 \end{array} \right. \end{array} $
	$ \begin{array}{c} R_1 \\ \hline \left. \begin{array}{l} \text{phenyl} \\ \text{cyclohexyl} \end{array} \right\} -CN-CH_2-N \begin{array}{l} \left\{ \begin{array}{l} H, H \\ H, R_4OH \end{array} \right. \\ \\ \left. \begin{array}{l} \alpha \text{ or } \beta\text{-naphthyl} \\ \text{p-diphenyl} \end{array} \right\} -CN-CH_2-N \begin{array}{l} \left\{ \begin{array}{l} R_3 \\ R_4 \end{array} \right. \\ \\ C_6H_5-CN-CH-CH_2-N \begin{array}{l} \left\{ \begin{array}{l} H, R_4 \\ R_3, R_4 \end{array} \right. \\ \\ R_2 \end{array} \end{array} $
Depressant only	

When the succinamide of one of the compounds (TT-131) was prepared in an attempt to increase the solubility, the activity of the base was lost entirely. In one instance (TT-161), the amino group was shifted one carbon further from the tetrazole nucleus by the insertion of an aminoethyl group, but no change in activity over the methyl derivative was observed.

SUMMARY

The effect on the central nervous system of 38 derivatives of aminomethyl tetrazole has been studied. This group of compounds falls into 3 distinct classes with regard to pharmacologic action, namely: convulsant, sedative, and those containing a mixture of both properties. A correlation of structure with pharmacologic activity has been attempted.

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STUDIES WITH TETRAZOLE DERIVATIVES

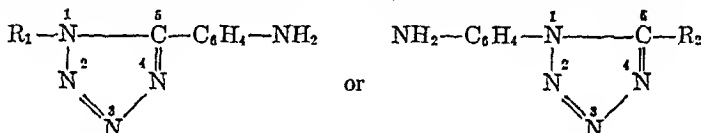
V. SOME PHARMACOLOGIC PROPERTIES OF AMINOPHENYL TETRAZOLES¹

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In the first four papers of this series (1-4) the relative convulsant, analeptic, and sedative actions of various types of tetrazole derivatives have been reported. In the progression of compounds from pentamethylene tetrazole (metrazol), through substituted tetrazoles, aminotetrazoles, and aminomethyltetrazoles, there was discovered a loss of analeptic action, a decrease in other stimulant actions, and the further development of depressant action. This fifth paper of the series is a report of some pharmacologic properties of 14 aminophenyl tetrazoles, in which an aminophenyl group occurs in positions 1 or 5 with one of a series of aliphatic groups at the only other possible point of substitution.



The structures of the substituted aminophenyltetrazoles were varied systematically and changes in pharmacologic activity were noted. The outline of systematic alterations of substituent groups and detailed reports of observations on animals are presented in table 1. None of these compounds to our knowledge has been previously described. General procedures were the same as those used in the previous papers.

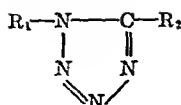
DISCUSSION. The insertion of a m-aminophenyl group in either the 1 or 5 position of tetrazole results in compounds having primarily depressant actions, although a convulsant factor seems to remain in a few cases. The presence of the p-aminophenyl group in either position produces compounds with almost no action in doses as high as 1000 mg./kg.

The size of the aliphatic side chain appears to be the chief influencing factor in the response pattern to the m-aminophenyl substituted compounds. Those having a methyl or an ethyl group in the R₁ position (TT-82, TT-028) give only anesthesia, whereas compounds with larger groups in this position (TT-076 to TT-034) tend to have less depressant action and a convulsant factor enters the pattern.

On the other hand, when the aliphatic side chain is in the R₂ position of the m-aminophenyl substituted compounds (TT-74 to TT-078) no convulsant factor enters the picture until the butyl group is reached (TT-086). Thus it appears that the greater depressant action may be obtained in this series by having the m-aminophenyl group attached to the nitrogen of the tetrazole nucleus.

¹ These compounds were prepared by E. Billhuber, Inc.

TABLE 1



COMPOUND NUMBER*	R ₁	R ₂	GENERAL ACTIONS	COMMENTS†
TT 66	CH ₃	p-NH ₂ -C ₆ H ₄	No action	Maximum dose 1000 mg/kg
TT 82	CH ₃	m-NH ₂ -C ₆ H ₄	Anesthesia	100 mg/kg, heavy sedation, 1000, anesthesia. Some analgesia. Rabbit IV 200, light anesthesia for 15 minutes
TT-028	C ₆ H ₅	m NH ₂ -C ₆ H ₄	Anesthesia	50 mg/kg, sedation, 500, anesthesia
TT-076	n C ₆ H ₇	m NH ₂ -C ₆ H ₄	Anesthesia with tremors	200 mg/kg, sedation, 500, anesthesia, latent tremors
TT-084	i C ₆ H ₇	m NH ₂ -C ₆ H ₄	Heavy sedation with tremors	200 mg/kg, mild sedation, 500, heavy sedation with intermittent tremors
TT-088	n C ₆ H ₅	m NH ₂ -C ₆ H ₄	Heavy sedation with tremors	20 mg/kg, sedation, 500, light anesthesia, latent convulsions
TT-034	i C ₆ H ₅	m-NH ₂ -C ₆ H ₄	Heavy sedation with tremors	30 mg/kg sedation, 100, heavy sedation, muscular tremors, 1000, fatal
TT 68	p-NH ₂ -C ₆ H ₄	CH ₃	No action	Maximum dose 1000 mg/kg
TT-74	m NH ₂ -C ₆ H ₄	CH ₃	Anesthesia	100 mg/kg, sedation, 400, anesthesia, Rabbit IV 150-300, immediate rigidity followed by anesthesia of short duration
TT-022	m NH ₂ -C ₆ H ₄	C ₆ H ₅	Anesthesia	50 mg/kg, sedation 400, anesthesia of several hours duration. Rabbit orally 250 mg/kg. No action
TT-082	m NH ₂ -C ₆ H ₄	n C ₆ H ₇	Anesthesia	100 mg/kg, sedation, 300, anesthesia IV 80, immediate anesthesia of short duration, larger doses increased duration. Orally 200, sedation, 600, anesthesia of long duration. Rabbits IV 25, slight sedation, 50, anesthesia for 5 minutes
TT-080	m NH ₂ -C ₆ H ₄	i C ₆ H ₇	Anesthesia	100 mg/kg, sedation, 200, anesthesia, 500, death. Orally 450, no sedation
TT 086	m NH ₂ -C ₆ H ₄	n C ₆ H ₅	Heavy sedation with tremors	100 mg/kg, sedation, 250, heavy sedation, IV 40, light anesthesia, prompt recovery, 50 anesthesia followed by convulsions, 80, fatal, Orally 200-500, slight sedation, Rabbit IV 35, anesthesia for 5 minutes
TT 078	m NH ₂ -C ₆ H ₄	i C ₆ H ₅	Anesthesia and convulsions	100 mg/kg, heavy sedation, 200, anesthesia, 500, death following tremors, Rabbit IV 80, heavy sedation followed by convulsions, respiratory difficulty, and death

* All compounds were used as the hydrochloride in aqueous solution, and were injected intraperitoneally unless otherwise noted

† Unless otherwise noted, all comments and doses concern albino rats of approximately equal weights

It is also apparent from this study that compounds having the amino group separated from the tetrazole nucleus by a phenyl group have greater depressant and less stimulant action than those compounds in which the phenyl is replaced by a methyl or an ethyl group (4).

A summary of the relations of chemical structure to pharmacologic activity of the five types of tetrazole derivatives in the entire series of papers is presented schematically in figure 1. In figure 1 an attempt has been made to correlate the character and trend in the action of the various tetrazole structures included in these studies. As might be anticipated the trends overlap to a certain degree.

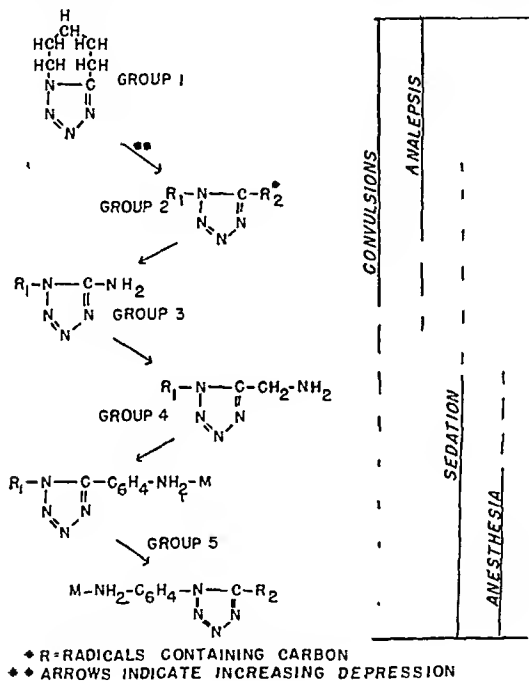


FIG. 1

Thus, in Group I, the alkylated pentamethylene tetrazoles, it was shown that a very high degree of stimulatory action could be attained by suitable substitution of the pentamethylene ring in the symmetrical position, but this action could be eliminated and even reversed to mild depression by suitable choice of the substituent (1). The compounds of this group also exhibited analeptic action but their potency as analeptics did not necessarily parallel their effectiveness as central nervous stimulants. Consequently, although this group of compounds was primarily stimulatory in its action, still depression appeared as a characteristic effect in a few instances. Only one compound showing a mixed effect was encountered.

The compounds of Group II, 1,5-dialkyl tetrazoles, exhibited either stimulatory or depressant effects, and mixed actions were not observed. In general, analeptic action paralleled the stimulatory action although there were several exceptions (2). In Group III, where the alkyl substituent in position 5 of the tetrazole ring had been replaced by an alkylated amino group, stimulation was the predominant effect; analeptic action had practically disappeared and depressant effects were only rarely observed (3). The compounds in Group IV, which differed from the preceding group in that the amino group was separated from the tetrazole ring by the interposition of one or two carbon atoms, generally exhibited a mixed action, the same compound eliciting alternating states of depression and stimulation (4). In a few instances stimulation alone unaccompanied by any obvious signs of depression was observed, while in other instances, notably when the substituent in position 1 was beta-naphthyl, depression without secondary stimulation was observed. It was not possible to predict the nature of the substituent groups which would cause elimination of one action and enhancement of the other. Finally in the Group V series, the results of which are summarized in this paper, the predominant action is depression, accompanied in only a few instances by a mild stimulatory action. Generally, stimulatory action, even if only slight, was noted in all groups although structural differences were rather marked, and conversely, depressive effects even if only on rare occasion made their appearance in all groups.

If the pentamethylene tetrazole derivatives of Group I are excepted in view of their unique structural characteristics as hetero-bicyclic systems with a hetero atom common to both rings, certain trends in pharmacologic action appeared to persist throughout all of the groups studied. Although the tetrazole ring system as such does not appear to be characterized by any pronounced pharmacologic action (2), the introduction of substituents into the 1 and 5 positions of the ring structure imparts definite and in many instances very profound actions to the resulting compounds. The new compounds uniformly exhibited central nervous system effects varying from marked stimulation to deep depression. The nature of the substituent groups, therefore, influenced greatly both the qualitative and quantitative aspects of the actions exhibited by these compounds. Even the choice and distribution of presumably inert alkyl groups often sufficed to alter the character of the pharmacologic response.

Perhaps the most interesting series of compounds from the point of view of variation in action is that identified as Group II (2), where both substituents are hydrocarbon radicals, either aliphatic, cycloaliphatic or aromatic and no functional groups are present. The action of the resulting compounds ranged all the way from profound stimulation, accompanied by marked analesis, to mild depression. An effort to correlate structural changes with changes in pharmacologic action in this group gives rise to the belief that substitution of similar groups in position 1 or 5 may cause opposite effects. In general it may be said that increasing the size of an alkyl substituent in position 1 will enhance the stimulatory action of a given compound, and, on the other hand, increasing the size of an alkyl substituent in position 5 will cause a decrease in the stimu-

latory action and eventually the disappearance of this effect later to be followed by the appearance of depressant action. One specific effect stands out, however, in that the cyclohexyl group, whether in position 1 or 5, will generally confer stimulatory action upon the compound but its effect is much more profound in the 1 position than in the 5 position.

A few specific examples will suffice to elucidate these trends. As noted above, tetrazole itself is apparently quite inactive. Disubstitution, as in 1,5-dimethyl tetrazole, introduces mild sedative action. When the methyl group in position 1 is replaced by a phenyl, iso-butyl or cyclohexyl group increasingly profound stimulatory action results. Further modification of the structure of the most potent stimulant of the group, 1-cyclohexyl-5-methyl tetrazole, by replacement of the methyl group in position 5 successively by ethyl and then normal and iso propyl or butyl groups, leads first to a marked decrease in stimulatory action and then to complete disappearance of this action. Thus maximum stimulatory action appears to reside in those compounds having a relatively large group in position 1 and a small group such as methyl in the 5 position. The substitution of large groups in both positions leads to complete loss of stimulatory action and frequently to the introduction of a mild depressant action. Even the cyclohexyl group, which in combination with a small group in either position bestows stimulatory action upon the resulting compound, seems to cancel its own effect when substituted in both positions as in 1,5-dicyclohexyl tetrazole.

Similar trends appear to exist in the other structural groups studied, although the qualitative nature of the action seems to be determined by the nature of certain functional groups present in these structures. Thus in the compounds of Group III, all of which carried an alkyl group in position 1 and an alkylated amino group in position 5 of the tetrazole system, the characteristic effect was central nervous stimulation. However, the action varied from mild stimulation to profound stimulation as the size of the alkyl group in position 1 increased from methyl to heptyl, maximum activity being reached with the five carbon chain. Modification of the substituent on the amino group effected further quantitative changes in the action of these compounds but did not appear to alter the character of the action. In both Groups II and III there was indication that the iso-butyl group and the cyclohexyl group when substituted in position 1 exerted approximately comparable effects upon the stimulatory potency of the tetrazole derivatives.

These general trends were again evident in the compounds included in Group V where an aminophenyl group was substituted in position 1 or 5 of the tetrazole ring and the unoccupied position was assumed by an alkyl group. Increasing size of an alkyl group in position 5 was generally accompanied by increased potency of these compounds as sedatives. A similar effect was observed with the isomeric series carrying the alkyl group in position 1. However, in addition a secondary convulsive action appeared in the compounds of this group and became more pronounced as the size of the alkyl group increased.

If the entire series of compounds, excepting the pentamethylene tetrazoles, is considered, it would appear in so far as hydrocarbon radicals as substituents

are concerned, that central nervous stimulation is favored by increasing the size of such a radical substituted in position 1 while depression is favored by increasing the size of an alkyl substituent in position 5. The introduction of a functional group such as the amino group as a substituent, either directly attached to the tetrazole ring in position 5, or separated from this point of attachment by one or more carbon atoms seems to be a determining factor in the character of the action exhibited by the resulting compounds. Directly attached to the tetrazole ring in position 5, compounds of purely stimulatory action result. When one or two carbon atoms are interposed between the amino group and the tetrazole ring, still at position 5, a mixed action results, the degree of stimulation or depression being modified by the nature of the substituent in position 1 or by the nature of substituents on the amino group (4). Interposing a cyclic system between the amino group and the tetrazole ring endows the resulting compounds with a depressant action, the action being more purely depressant when the aminophenyl group occupies position 1 than when it occupies position 5.

SUMMARY

The action of a group of aminophenyltetrazole derivatives on the central nervous system has been studied. The presence of a m-aminophenyl, but not the p-aminophenyl group, in either the 1 or 5 position of the tetrazole nucleus produced compounds having primarily depressant actions, depending upon the size of the aliphatic side chain. A schematic diagram of the relation of structure to pharmacologic activity in the 130 compounds of the complete series (1-4 and Group 5) is presented and discussed.

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INFLUENCE OF THE AUTONOMIC NERVOUS SYSTEM ON THE INTESTINAL ABSORPTION OF A HYPERTONIC SOLUTION OF GLUCOSE

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The absorption of glucose by the small intestine has been studied extensively. Nevertheless, very little is known about the influence of the autonomic nervous system upon this process, and the results of the few studies which have been made are contradictory.

Kotschneff (1) stated that in the dog adrenalin injected subcutaneously prevents the absorption of glucose. Van Liere, Northrop and Sleeth (2) reported the same finding. Gellhorn and Northrop (3) found that a small concentration of adrenalin reduces the absorption of glucose in the intestine of the frog, while greater concentrations increase the absorption. According to these authors (4), acetylcholine has the opposite effect. W. Borchardt (5) found that denervation of the intestine in the dog did not alter the absorption. Gellhorn and Northrop (6) were unable to obtain satisfactory results with the stimulation of the splanchnic and the vagus. Horne, McDougall and Magee (7) showed that in rabbits and rats acute denervation increases the absorption of glucose; in rabbits, however, section of the splanchnic three weeks previously did not alter the quantity of glucose absorbed. On the other hand, section of the splanchnic only ten days previously increased the absorption by 90%. Section of the vagus also increased the quantity of glucose absorbed. These same authors deny that atropine affects the absorption of glucose, adding that the role of the autonomic nervous system in this function is not clear. Lajos (8) states that atropine slows the rate of absorption of glucose in rats.

The object of the present work is to study certain aspects of the action of the autonomic nervous system in the absorption of a hypertonic solution of glucose, and to observe the effect on this process of certain drugs which modify autonomic activity.

METHODS Unselected adult cats were used which had fasted for at least twelve hours before the experiment was begun. They were anesthetized with sodium pentobarbital (Nembutal, Abbott). 0.033 gm was dissolved in 1 cc of 25% urethane and 1.2 to 1.4 cc of this solution per kilogram of body weight was injected intramuscularly. A 10% solution of glucose (Sehering-Kahlbaum) was used. Four cc of this solution was introduced into a loop of the small intestine by the following technique: an opening was made in the free border of the jejunum 1 cm below the ligament to which its upper end is attached, and through this opening a tube 16 cm in length and 3.5 mm in external diameter was introduced towards the distal portion. Immediately below the free end of the tube the intestine was ligated and again immediately below the opening. In this way a loop of the intestine was isolated which was always of the same length and in which the blood supply remained

intact. The abdominal wall was closed and the intra-abdominal temperature was maintained between 37° and 38°C.

The experiments were begun by washing out the loop of intestine with a 10% solution of glucose. When this had been done 4 cc. of the solution was left in the loop. The contents of the loop were removed 5, 10, 15, 20, 30, 40, 50 and 60 minutes after the beginning of the experiment, the volume was measured, a 0.3 cc. sample was placed in a test-tube for estimation of glucose, and the liquid was returned to the intestinal loop. These procedures did not require more than 30 seconds. In view of the difficulty of recovering all of the solution contained in the intestinal loop, the error of the method was calculated statistically. No significant difference was found between the volume introduced and the volume recovered.

When the splanchnic or vagus was stimulated, electronically controlled condenser discharges were employed. Silver electrodes insulated with rubber and absorbent cotton were used. The strength of the stimulus was maximal or slightly supramaximal. At the end of the experiment the position of the electrodes and the condition of the nerve were verified. The left greater splanchnic nerve was stimulated, the proximal portion being crushed. In some experiments, the adrenal on the same side was ligated. In the case of the vagi, the two nerves (superior and inferior) were stimulated, the central portion traversing the diaphragm being crushed. Artificial respiration was used in this type of experiment.

The glucose content of the solution was estimated by the method of Solomo (9), adapted in the following way: 0.1 cc. of the sample was diluted to 10 cc. with distilled water; this solution was added to the solution of ferricyanide which had been brought to a boil. Statistical calculation showed that there was no significant difference between the theoretical quantity of glucose in the samples and that found by analysis with this method.

The drugs employed were: atropine sulfate (Merek), neostigmine (Prostigmine, Roche) and ergotamine (Gynergen, Sandoz). Doses and routes of administration are indicated below.

RESULTS. Three types of experiments were carried out: I) animals injected with atropine, prostigmine and ergotamine; II) animals in which the splanchnic was stimulated; and III) animals in which the abdominal vagus was stimulated. For the sake of greater clarity, each type of experiment will be described separately.

I) Animals injected with atropine, prostigmine or ergotamine. In this experimental series, as in the others, we may consider two types of results: 1) modifications under different experimental conditions in the changes in volume of the 4 cc. of 10% glucose, and 2) the intestinal absorption of glucose.

a) Control animals. It is known that hypertonic solutions in the lumen of the intestine tend to become isotonic through the passage of fluid into the lumen. As a result, the 4 cc. of glucose solution increases in volume during the first 30 minutes of the experiment and then the volume diminishes as indicated in Fig. 1, which represents the mean of 11 experiments.

Parallel with the changes in volume glucose is absorbed. For the first 15 minutes the absorption is rapid, and for the remaining 45 minutes it is slower, the original curve becoming a straight line with less marked slope (see Fig. 2).

b) Action of atropine. When atropine (0.001 gm./kg.) is injected intravenously immediately before the beginning of the experiment, the volume of the original 4 cc. of 10% glucose solution placed in the loop of intestine is altered significantly after the first 20 minutes (Fig. 1, which shows the mean values for 8 animals). The curve representing the changes in volume is lower than the con-

trol curve, reaching the greatest dilution 25 minutes after the start of the experiment.

Atropine does not cause any significant alteration in the curve for the absorption of glucose (Fig. 2).

c) Action of prostigmine. Prostigmine injected intramuscularly in a dose of 0.0005 gm./kg. 10 minutes before the start of the experiment altered the curve of the changes in volume in the opposite direction to atropine. (See Fig. 1, which presents the mean of 9 experiments.) It is seen that prostigmine increases considerably the volume of the solution, which reaches a maximum at 35 minutes.

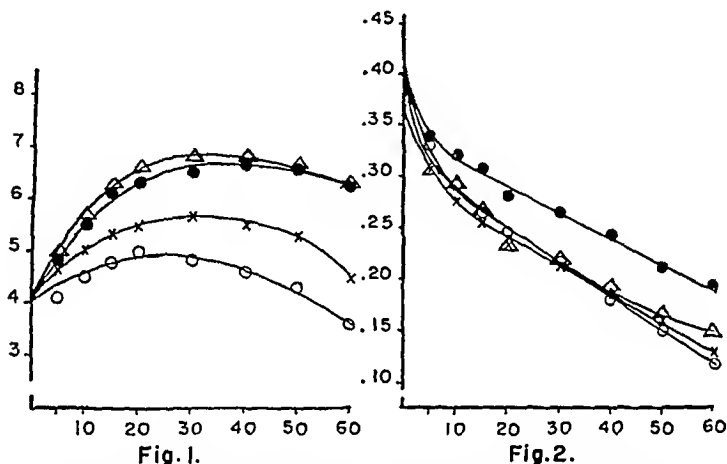


FIG. 1. CHANGES IN VOLUME OF 4 CC. OF 10% GLUCOSE SOLUTION PLACED IN A LOOP OF SMALL INTESTINE. ORDINATES: CC.; ABSCISSA: TIME IN MINUTES

× Curve obtained in cats anesthetized with Nembutal.

○ Same, but with previous intravenous injection of atropine, 0.001 gm./kg. of body weight.

● Same as control, but with intramuscular injection of ergotamine, 0.002 gm./kg. of body weight, 10 minutes before the beginning of the experiment.

△ Intramuscular injection of prostigmine, 0.0005 gm./kg. of body weight, 15 to 25 minutes before the beginning of the experiment.

FIG. 2. ABSORPTION OF GLUCOSE FROM THE 4 CC. PLACED IN THE LOOP OF INTESTINE. ORDINATES: GRAMS OF GLUCOSE; ABSCISSA: TIME IN MINUTES

Remainder of legend same as in Fig. 1

Even at 10 minutes there is a significant difference between the two curves. The quantity of glucose absorbed in the presence of prostigmine is no different from that absorbed under normal conditions (Fig. 2).

d) Action of ergotamine. Ergotamine injected intramuscularly in a dose of 0.002 gm./kg. 15 to 25 minutes before the start of the experiment caused a marked alteration in the curve of the volume changes of the glucose solution. Like prostigmine, ergotamine increased the volume of the intestinal contents above the control (Fig. 1).

As indicated in Fig. 2 (mean of 8 animals), ergotamine alters the intestinal

absorption significantly, but does not alter the form of the absorption curve. At the start the absorption is rapid, then slower, becoming a linear function. The entire curve is parallel to the normal absorption curve.

During the entire course of the experiments the effect of prostigmine and ergotamine could be observed in other organs: eyes, salivary glands, muscles, etc.; from which we conclude that the action of the drugs continued throughout the experiments and that the dose was effective. The action of atropine is known to be prolonged.

II) *Animals in which the splanchnic was stimulated.* a) Control animals. In these animals electrodes were placed on the left greater splanchnic nerve and the

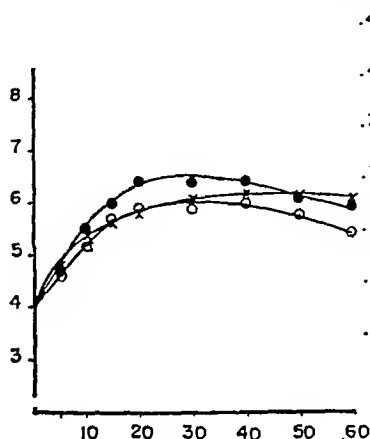


Fig. 3.

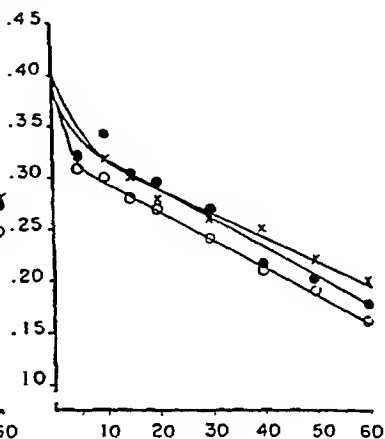


Fig. 4.

FIG. 3. CHANGES IN VOLUME OF 4 CC. OF 10% GLUCOSE SOLUTION PLACED IN A LOOP OF SMALL INTESTINE. ORDINATES: CC.; ABSCISSA: TIME IN MINUTES

× Curve obtained in cats anesthetized with Nembutal in which electrodes were placed upon the left greater splanchnic with the proximal portion of the nerve crushed.

● Same as above, but with the left splanchnic stimulated maximally at approximately 200 per minute.

○ Same as above, but with the left adrenal ligated.

FIG. 4. ABSORPTION OF GLUCOSE FROM THE 4 CC. PLACED IN THE LOOP OF INTESTINE. ORDINATES: GRAMS OF GLUCOSE; ABSCISSA: TIME IN MINUTES

Remainder of legend same as in Fig. 3

nerve was crushed above them. An appreciable change in the volume curve for the glucose solution was observed as compared with the control animals of the first series. It is seen that crushing the splanchnic causes a greater dilution, a difference which is observed at about 40 minutes. Subsequent reduction in the volume is very slight. The intestinal absorption is also altered, and in this case the quantity of glucose absorbed is significantly less than that in the control animals of series I; nevertheless the form of the curve is the same. In the beginning the absorption is rapid, and then becomes slower. After 15 minutes the two curves are practically parallel. (Figs. 3 and 4 represent the mean for 6 animals.)

b) Stimulation of the splanchnic. Stimulation of the splanchnic for one hour at a frequency of approximately 200 per minute alters the curve of the volume changes as indicated in Fig. 3. It is seen that at first the volume becomes greater than the control, reaching maximal dilution at 30 minutes. The subsequent reduction in volume is also greater.

Stimulation of the splanchnic gives a glucose absorption curve which is little different from that obtained in control animals, as seen in Fig. 4.

c) Stimulation of the splanchnic after adrenalectomy. In order to determine the role of the adrenals in the phenomena described, the adrenal gland was ligated on the side on which the splanchnic was stimulated, while the other conditions of the experiment were kept constant. Stimulation of the splanchnic after ligation of the adrenal caused the curve of the changes in volume of the 4 cc. to diverge from the control curve only after 30 minutes from the beginning of the experiment. In this case, as in the case of stimulation of the splanchnic with the adrenal intact, the volume began to decrease markedly (see Fig. 3). If the curve of the volume changes is compared in animals in which the splanchnic is stimulated with the same curve in animals in which the adrenal is ligated, it can be seen that the latter is lower than the former and that both are of the same form. The influence of the adrenal during the stimulation of the splanchnic is evident in the absorption of glucose, especially in the first minutes, as is shown in Fig. 4.

The difference in the absorption between the control animals and those in which the splanchnic is stimulated and the adrenal ligated is significant.

III) *Animals in which the abdominal vagus was stimulated.* a) Control animals. In this series of experiments it was necessary to administer artificial respiration to all the animals. The results in control animals do not differ much from the controls of series II so far as the changes in volume are concerned (see Fig. 5, which shows the mean values for 6 animals). By comparing the curve of volume changes in the control animals with that obtained in section I, it is clear that the greatest difference is due to the lack of reduction of the volume in the controls. Thus although the curves are identical up to 25 minutes, the difference after this period is considerable, since in series I the volume slowly decreases, while in series III it does not. The absorption of glucose in this series, as in the majority of the curves obtained, is more rapid during the first 15 minutes and slower during the remainder of the experiment, as is seen in Fig. 6.

b) Stimulation of the vagi. Two series of experiments were carried out to determine whether different frequencies of stimulation have any effect on the changes in volume of the 4 cc. of solution introduced into the intestine or on the absorption of glucose. In one series, the nerves were stimulated for an hour at a frequency of approximately 200 per minute; in the other they were stimulated for an hour at 25 per second. Since there was no significant difference between the results, a mean value for the two series of experiments was taken.

The changes in volume undergone by the 4 cc. at the beginning of the experiment reproduce a dilution curve which is practically the same for the first 30 minutes as the curve obtained with prostigmine (Section I,c); later the curves

diverge somewhat because the stimulation of the vagi prevents a later reduction in volume.

In comparison with its control the change in volume caused by stimulation of the vagus is very slight, representing a significant difference at only two points (see Fig. 5, which shows the mean values for 12 animals).

Stimulation of the vagus causes an increased absorption of glucose at the end of the experiment, when there is a significant difference from the control.

c) Crushing the vagi. Crushing the vagi at their entrance into the abdomen causes no important modification in the changes in volume of the solution of glucose (Fig. 5, mean value for 6 animals); on the other hand, it has a definite

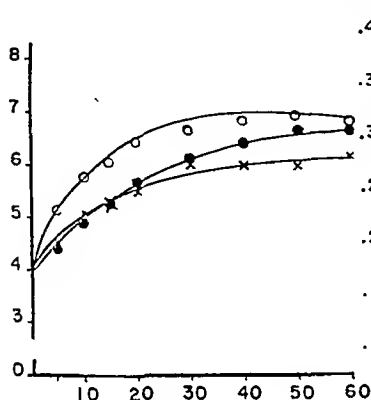


Fig. 5.

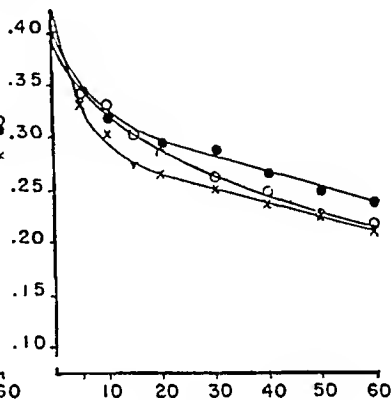


Fig. 6.

FIG. 5. CHANGES IN VOLUME OF 4 CC. OF 10% GLUCOSE SOLUTION PLACED IN A LOOP OF SMALL INTESTINE, WITH STIMULATION OR CRUSHING OF THE ABDOMINAL VAGI. ORDINATES: CC.; ABSCISSA: TIME IN MINUTES

- Control.
- Curve obtained during stimulation of the vagi.
- × Curve obtained after crushing the vagi.

FIG. 6. ABSORPTION OF GLUCOSE FROM THE 4 CC. PLACED IN THE LOOP OF INTESTINE. ORDINATES: GRAMS OF GLUCOSE; ABSCISSA: TIME IN MINUTES

Remainder of legend same as in Fig. 5

effect upon intestinal absorption: section of these nerves causes significant increase in the quantity of glucose absorbed (Fig. 6).

DISCUSSION. While it is true that a great number of authors declare that the absorption of glucose in the small intestine is a linear function and is thus independent of the concentration and of the volume (for the literature see Verzá and McDougall, (10)), nevertheless there are other authors who are unable to demonstrate this relationship between the quantity of glucose absorbed and time (Ravdin, Johnston and Morrison, (11)).

Our control experiments show that the quantity of glucose absorbed from 4 cc. of a 10% solution placed in the intestine is linear only after the first 15 minutes of the experiment; during the first moments the absorption is very rapid, giving a curve of greater slope than the later straight line function.

The curve referred to (control curve, Fig. 2) undergoes these changes when the glucose solution becomes isotonic. Actually it becomes linear when the concentration of glucose is between 5.4 and 4.7%; these results do not agree with those reported by some authors (see Groen (12), and Verzár and McDougall, (10)). Furthermore this would indicate that the rate of absorption of glucose is greater when the solution is hypertonic than when it is normal or isotonic; hypertonicity above 10% is unnecessary to cause more rapid absorption (see the authors cited).

The experiments in which electrodes were placed on the vagi which were crushed centrally may be considered as experiments in which an acute section of these nerves was made, since the electrodes which are fixed in place do not stimulate the nerves. It is evident then that section of the vagi has a considerable influence upon the absorption of glucose, a finding which is in accord with the report of Horne, McDougall and Magee (7). The group of experiments in which the left greater splanchnic nerve was crushed obviously cannot be considered as acute total sections of the sympathetic system.

When the splanchnic and the vagi were stimulated frequencies of either 200 per minute or 25 per second were used. With these frequencies of stimulation there is no fatigue of the preparation (Orias (13)). Furthermore the intensity of the stimulus was maximal or slightly supramaximal. Therefore it is believed that the stimulation employed in all these experiments was effective at all times. Thus our results are not in accord with those obtained by Gellhorn and Northrop (6), who found no constant effect of stimulation of the vagus or of the splanchnic upon the absorption of glucose.

In studying the various curves one's attention is called particularly to the curve showing the absorption of glucose when the left splanchnic is stimulated without ligation of the adrenal on the same side (Fig. 4). In it there are certain points which diverge from the curve, but we are unable to interpret this phenomenon.

It is interesting to recall two facts from these experiments: 1) the changes in volume undergone by the 4 cc. of 10% glucose solution and, 2) the phenomena of intestinal absorption of glucose contained in this solution.

1) The results reported above are in perfect accord: both excitation of the vagus and prostigmine dilute the initial volume; but atropine by inhibiting the cholinergic fibres of the vagus causes less dilution in comparison with the control. In the same way ergotamine in paralyzing the adrenergic fibres disturbs the cholinergic-adrenergic equilibrium in favor of the cholinergic and then the initial 4 cc. increases in volume just as when one excites the vagus or injects prostigmine. Section of the vagi at their entrance into the abdomen cuts off the impulses which come from the higher centers and disturbs the equilibrium previously mentioned in favor of the adrenergic system, and it is for this reason that the curve of dilution obtained when the vagi were crushed is so similar to that obtained upon stimulation of the splanchnic.

All that has been said tends to show that the cholinergic system favors the dilution of the hypertonic solution of glucose.

If the parasympathetic system has an important influence upon the changes in volume of the 4 cc. of 10% glucose placed in the intestinal loop at the beginning of the experiment, the same is not true of the sympathetic system. In fact there is no greater significant difference in the changes in volume when the left splanchnic is stimulated whether or not the adrenal on this side is present.

2) The action of the autonomic system upon the absorption of glucose is not very clear. In fact only ergotamine and section of the vagi affect the absorption of glucose significantly; these findings are very difficult to correlate and would seem to contradict the findings of Borchardt (5) and of Horne, McDougall and Magee (7). Neostigmine and atropine do not affect the absorption in any way, contrary to the opinion of Lajos (8) who claims that atropine slows the absorption of glucose in the rat. Nor have differences been found when the vagi are stimulated or sectioned, a finding which coincides in part with the reports of Borchardt (5), Gellhorn and Northrop (6), and Horne, McDougall and Magee (7).

The comparison of the apparent action of the autonomic nervous system upon the phenomena of dilution of the hypertonic solution of glucose and the very slight importance which it has in the intestinal absorption of this substance leads one to suppose that the visceral nervous system of the abdomen dilutes the hypertonic solutions mainly by way of a mechanism of cellular hypersecretion.

The fact that the curves of absorption of glucose obtained in healthy animals (controls) and in animals under the influence of atropine and of prostigmine, coincide almost exactly, and furthermore the fact that the curves of dilution of the 4 cc. differ so much under the same experimental conditions would indicate that the absorption of glucose is a phenomenon which is independent of the concentration of the solution. This is in agreement with many authors (see Verzář and McDougall (10)).

SUMMARY

The changes in volume and the absorption of glucose from 4 cc. of a 10% solution of glucose placed in a loop of small intestine 16 cm. long with intact circulation have been studied under various conditions in cats anesthetized with Nembutal.

Normally there is an increase in volume with a later decrease (Fig. 1), while the glucose is absorbed rapidly during the first few minutes and later more slowly (Fig. 2).

Atropine prevents much increase in the original volume but does not affect the absorption of glucose.

Prostigmine and ergotamine increase the volume of the solution significantly; on the other hand while prostigmine does not affect the absorption of glucose, ergotamine reduces it significantly (Figs. 1 and 2).

Stimulation of the splanchnic with or without the adrenal does not alter the volume of the solution to an important degree. But while stimulation of the splanchnic with the adrenals intact does not change the absorption of glucose

significantly, stimulation of the splanchnic without the adrenal causes a significantly greater absorption of glucose (Figs. 3 and 4).

Acute section of the abdominal vagi does not change the absorption of the fluid from the intestinal loop; on the other hand it promotes the absorption of glucose to a significant extent. Stimulation of these nerves causes a greater increase in the volume and also alters the absorption of glucose significantly (Figs. 5 and 6).

We are grateful to Messrs. LaRoche and to Messrs. Sandoz for the Prostigmine and the Gynergen furnished for use in this investigation.

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EFFECT OF ACIDOSIS AND ANOXIA ON THE CONCENTRATION OF QUINACRINE AND CHLOROQUINE IN BLOOD*

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The extensive variation in plasma quinacrine concentration found among individuals adhering to a specified dosage intake was recognized by Shannon *et al.* (1) in their early studies on the physiological disposition of the drug. The extent of variability in man is indicated in data obtained at Ft. Knox (2). On a carefully supervised dosage regimen there was a 7-fold difference between the drug levels which delimited the 95 per cent range.

The factors underlying such wide fluctuations in individual plasma levels are obscure. Marshall and Dearborn (3) and Dearborn (4) showed in animals that tissue concentrations of quinacrine are more uniform on any given dosage regimen and are more closely correlated with dosage intake and with the suppressive effect in avian malaria. They directed attention to the distribution of drug between tissue and plasma as the major source of variability since the relative concentration in tissue is so high.

In the present study two factors were examined which were considered likely to play a role in the partition of drug between tissues and plasma, namely the acid-base balance of blood and the degree of oxygenation. It was previously shown that oral ingestion of acidifying and alkalinizing salts exerts a profound influence upon the extent of urinary excretion of quinacrine, chloroquine and santoquine (5). Such changes in rate of elimination may be reflected secondarily in the plasma level. The present work is concerned not with such secondary changes but rather with an altered partition of quinacrine and chloroquine between tissue and plasma.

METHODS Quinacrine and chloroquine, in aqueous solution, were administered to dogs by stomach tube. Daily doses were given for periods of 2 to 18 days, the last dose was given 24 hours before the experiment. The animals were anaesthetized about 1 hour before the experiment with sodium phenobarbital given intraperitoneally (140 mg per kg) or intravenously (65 mg per kg, with additional supplements). A total of 12 dogs were used, 10 treated with quinacrine and 2 with chloroquine.

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TABLE 1

Effect of carbon dioxide and anoxia on blood concentration of quinacrine and chloroquine

DOG NO., DRUG, AND DOSAGE	EXPERIMENTAL PROCEDURE	PLASMA DRUG	RED CELL DRUG	WHOLE BLOOD DRUG	BLOOD pH	CO ₂ CON- TENT	O ₂ CON- TENT	DURATION OF TREATMENT
		$\mu\text{gm./l.}$	$\mu\text{gm./l.}$	$\mu\text{gm./l.}$		vol. %	vol. %	min.
Dog No. 6, Quin- acrine, 25mg./ kg./day, 3 days	Control	59	217	1450	7.4	45	20	150
	CO ₂	142	440	1725	6.8	62	22	105
	Recovery	51	205	1370	7.3	48	19	110
	Anoxia	52	190	1250	7.3	40	8	130
Dog no. 7, Quin- acrine, 15 mg./ kg./day, 12 days	Control	82	76	1350	7.3	48	17	Prean*
	Control	105	124	1130	7.4	43	17	60
	CO ₂	135	184	1460	6.9	67	18	80
	Recovery	66	100	1170	7.3	53	17	145
Dog no. 4, Quin- acrine, 20 mg./ kg./day, 2 days	Anoxia	77	120		7.3	44	8	85
	Control	57	147	718	7.5	44	19	150
	CO ₂	176	375	1144	6.8	79	23	70
	Recovery	70	147	745	7.5	39	19	125
Dog no. 5, Quin- acrine, 40 mg./ kg./day, 2 days	Anoxia	114	275	891	7.2	44	1.4	60
	Control	100	158	1465				120
	CO ₂	212	343	1790				60
Dog no. 2, Quin- acrine, 20 mg./ kg./day, 14 days	Control	111	210	570				60
	Control	105	220	500				85
	CO ₂	210	480	580				60
	Recovery	85	180	580				120
Dog no. 3, Quin- acrine, 20 mg./ kg./day, 14 days	Anoxia	140	300	500				40
	Control	45	140	430	7.2	59	17	70
	Anoxia	360	900	940	6.5	97	1.0	100
Dog no. 8, Chloro- quine, 15 mg./ kg./day, 9 days	Control	1900						Prean.*
	Control	1960	1500	5000	7.2	56	10	60
	CO ₂	3560	2600	8100	6.9	65	14	60
	CO ₂	2520	2150	10000	6.9	65	13	120
Dog no. 1, Chloro- quine, 10 mg./ kg./day, 2 days	Recovery	1900	2400	8300	7.2	54	13	135
	Anoxia	1560		10300	7.1	54	7	130
	Control	113	470	2800				90
	CO ₂	230	1160	2800				30
Dog no. 1, Chloro- quine, 10 mg./ kg./day, 2 days	Recovery	93	200	2700				165
	Anoxia	130	245	2400				70

* Preanaesthesia.

Gases were administered by tracheal cannula connected to a closed circuit in which circulation was maintained by a motor blower. The closed system consisted of a 100-liter spirometer, a 5-liter spirometer, and a CO₂-absorber all in series with the blower. In the

CO₂ experiments, the large spirometer was filled with a mixture of 10 per cent CO₂-90 per cent O₂ and the soda-lime absorber was removed. In the anoxia experiments the large spirometer was partially filled with air and the CO₂-absorber was retained, so that the O₂-tension fell gradually as a result of re-breathing.

Arterial blood samples for drug analysis were obtained by cardiac or arterial puncture with potassium oxalate as the anticoagulant. Samples for gas analysis and pH measurement were drawn under oil from the femoral artery with heparin as the anticoagulant.

O₂ and CO₂ content were measured by the manometric method of Van Slyke (6). The pH of whole blood was estimated at 38°C. with a syringe type of glass electrode.

Quinacrine was estimated by the single extraction procedure of Brodie and Udenfriend (7) with the modification introduced by Masen (8) of acidifying with trichloroacetic acid instead of acetic acid. In most plasma measurements the alkaline wash was omitted; it was retained in all tissue analyses. Chloroquine was measured by the ultra-violet irradiation method of Brodie and Udenfriend (9). Tissue samples were homogenized in the Waring blender in N/20 HCl.

TABLE 2

Rate of increase of plasma quinacrine during carbon dioxide administration

DOG NO. AND DOSAGE	ANALYTICAL METHOD	INITIAL	12 MIN.	60 MIN.	100 MIN.	RECOVERY
		μgm./l.	μgm./l.	μgm./l.	μgm./l.	μgm./l.
Dog no. 11, 20 mg./kg./day, 14 days	Alkali washed	140*	240	300	440*	140*
	Unwashed	200	320	420	560	200
Dog no. 12, 20 mg./kg./day, 18 days	Alkali washed	140*	200	250*		
	Unwashed	240	290	390		

Tissue samples were taken at times indicated by * and results are recorded in table 3

Since the wash with 10 per cent NaOH removes certain degradation products and confers increased specificity upon the quinacrine analyses, comparative measurements were made on all plasma samples of Dog No. 11 and No. 12, with and without the alkaline wash (table 2). The wash with alkali reduced the amount of fluorescent material read as quinacrine by about 25 per cent. Since the reduction was consistent in samples taken before and during CO₂ administration the wash did not change the trend of the results. Thus, the same phenomenon occurred with both quinacrine and its degradation products.

RESULTS. The general pattern of each experiment consisted of four periods: (1) control period with the animal breathing air; (2) CO₂ period during which 10% CO₂ in O₂ was administered; (3) recovery period with the dog again breathing air; (4) anoxia period during which the O₂ tension of the inspired air fell progressively. Experiments were carried out on 12 dogs. Illustrative records are presented in table 1.

Two separate control measurements on plasma drug level were obtained in each of 3 dogs. The two controls agreed well with a maximum difference of 25 per cent. Recovery levels were obtained in 6 dogs, and these approximated the initial control levels quite closely with a maximum difference of about 25 per cent. Measurements on the red blood cell concentrations were more erratic, but nevertheless consistent changes were obtained.

Drug concentration in plasma and in red blood cells rose to about double the initial control value during administration of 10 per cent CO₂ in O₂ over a period

of 1 or 2 hours (table 1). This result was obtained uniformly in all the animals and the magnitude of change was well beyond the range of spontaneous fluctuations. Initial levels were higher in red blood cells than in plasma, but the percentage increase under CO_2 was about the same. The drug levels in both plasma and erythrocytes returned to normal during the 2-hour recovery period.

The concentration of drug in whole blood usually rose about 25 per cent during the CO_2 period. It is significant that the percentage elevation of drug level in whole blood is far less than that obtained in plasma or in red cells. The elevation obtained in whole blood can be completely accounted for by the increased drug in plasma and in erythrocytes. In addition, there was encountered a rising leucocyte count amounting to about 10 or 20 per cent increase during each experimental period which in itself would elevate the whole blood level. Since leucocytes carry about three-fourths (1) of the drug present in whole blood, it is evident that they do not participate in the 100 per cent elevation of the concentration of drug observed in plasma and in red blood cells. Within the large experimental error of this type of experiment it appears that the drug concentration within the leucocytes remains essentially unchanged during the administration of CO_2 . There could be a slight reduction that would not be detected because of the large errors.

Time relationships of the response to CO_2 are indicated in two dogs, No. 11 and No. 12 (table 2). A definite rise in plasma quinacrine is apparent at 12 minutes with further increase at 60 and 100 minutes. Data are not at hand with which to evaluate any further trend.

It should be noted further in the experiments of table 2 that the plasma drug response follows the same course whether or not the analytical procedure includes an alkaline wash. Within the limits of specificity conferred upon the method by the alkaline wash, the major portion of the plasma drug elevation is contributed by quinacrine and not by some alkali soluble degradation product.

Parallel measurements were made upon blood pH and CO_2 content to evaluate the degree of acid-base disturbance. The pH of the blood dropped about 0.5 unit, from 7.4 to 6.9. CO_2 content rose about 20 or 30 volumes per cent from an initial value of 45 volumes per cent. The animals displayed full oxygenation during the CO_2 -period.

The concentration of drug in muscle tissue showed no measurable response to CO_2 (table 3) in two dogs, No. 11 and No. 12. Plasma levels in these dogs showed the characteristic elevation (table 2). Control measurements on corresponding muscle strips taken from the same muscle groups of the right and left limb showed agreement within about 15 per cent. If the control strips were taken at the beginning of the experiment and the corresponding muscle strips of the opposite limb were excised at the close of the CO_2 -period the drug concentration still agreed within the experimental error. In dog No. 11, three strips of gluteus muscle, one taken as a control before CO_2 , one taken after 100 minutes of CO_2 -administration, and the third taken after a 2-hour recovery period, all showed good agreement in tissue drug concentration. Drug concentration in muscle remained essentially unchanged during CO_2 -administration.

The influence of anoxia can be correctly evaluated only if data are at hand concerning concomitant changes in blood pH. In dog No. 6 (table 1), in which the pH of the blood had remained constant, anoxia induced no change of drug concentration in plasma or red cells. In dog No. 4, anoxia was accompanied by a drop in blood pH from 7.5 to 7.2; the drug level in plasma and in red blood cells rose to an extent consistent with the acidosis. In dog No. 3, anoxia was associated with severe respiratory failure accompanied by a marked drop in blood pH and with great accumulation of CO_2 . The elevation of drug level was about 8-fold in plasma and erythrocytes, and about 2-fold in whole blood. One may conclude that anoxia in itself has little or no influence upon drug concentration in the various elements of the blood. If anoxia is accompanied by acidosis the change in drug level is consistent with the pH-drop. However, in extreme asphyxia, as in dog No. 3, there may be some additive effect attributable to anoxia.

TABLE 3

Effect of carbon dioxide on the concentration of quinacrine in muscle tissue

DOG NO. 11			DOG NO. 12		
Treatment	Muscle	Quinacrine	Treatment	Muscle	Quinacrine
		mg./kg.			mg./kg.
Control	L. hamstring	8.4	Control	L. hamstring	8.9
Control	R. hamstring	9.0	Control	R. hamstring	7.8
Control	R. brachial	7.0	CO_2	R. hamstring	7.6
CO_2	L. brachial	7.0	Control	L. gluteus	10.0
Control	L. gluteus	13.4	CO_2	R. gluteus	12.0
CO_2	R. gluteus	12.4			
Recovery	R. gluteus	12.6			

DISCUSSION. Administration of CO_2 in amount sufficient to reduce blood pH from 7.4 to 6.9 resulted in a 100 per cent rise of drug concentration in plasma and in erythrocytes. Drug concentration in muscle tissue and in leucocytes remained essentially unchanged under the same conditions. It is highly probable that drug concentration in the parenchymatous organs such as liver, spleen and kidney similarly was unaffected. The two drugs under consideration, quinacrine and chloroquine, exhibit a distribution pattern characterized by a high concentration of drug in tissue relative to plasma. If the rise in blood concentration is due to a diffusion of drug out of the tissues, the total increase in circulating drug would cause but a negligible fall in tissue concentration. Consequently, it would appear that acidosis exerts its influence by altering the partition of drug between tissue and plasma, leaving the tissue drug concentration essentially unchanged and increasing the plasma level.

Changes in blood pH might be expected to influence the distribution of a weak organic base between the various phases of the body. Quinacrine and chloroquine are both basic compounds whose acid-base characteristics have been for-

mulated by Irvin and Irvin (10, 11). Each compound has two proton-accepting groups with one dissociation constant, pK'_1 , involving the aromatic nucleus, ($pK'_1 = 8.06$ for chloroquine; 7.67 for quinacrine) and the other, pK'_2 , representing the diethylamino side-chain ($pK'_2 = 10.16$ for each drug).

Although the amount of free base present at blood pH is minute, nevertheless it is highly probable that it is the relative concentration of undissociated base which determines the distribution of drug between plasma and the various tissue phases. In the partition of quinacrine between an aqueous buffer and an immiscible organic solvent, Craig's (12) experiments suggest that the free base is the species which is transferred and which establishes equilibrium between the two phases. However, it can not be disregarded that the relative concentration of monopolar cation may also be involved in the partition equilibria.

By a derivation essentially the same as is applicable to the dissociation of a dibasic acid, one can calculate the proportion of drug which is present in the form of free base. Using the terminology and constants of Irvin and Irvin (10, 11) we find:

$$\frac{\text{free base}}{\text{total drug}} = \frac{[B - B']}{[S]} = \frac{K'_1 K'_2}{[H^+]^2 + K'_1 [H^+] + K'_1 K'_2}$$

In the case of quinacrine this ratio is calculated to be 60×10^{-5} at pH 7.4 and 8×10^{-5} at pH 6.9. The degree of acidification encountered in the animal experiments would reduce the proportion of free base by a factor of eight. One might expect transfer of drug from tissue to plasma in order to elevate the concentration of free base toward its initial equilibrium level. However, there is little ground for quantitative prediction since we do not know the degree of acidification of the intracellular fluid nor do we know the nature of the equilibria which govern the accumulation of drug in certain tissues of the body. There are other variables which make it all the more difficult to describe the system responsible for the experimental results in the form of a simple expression. The decrease in plasma binding of quinacrine at lower pH levels (Taggart, 13) would constitute a factor opposing the shift of drug from tissues to plasma.

It is of interest to note the absence of an anoxic effect upon the drug partition. If the high partition ratio between the tissue and plasma were maintained by a metabolic process one might expect anoxia to exert a pronounced effect. In the experiments deep anoxia in the absence of acidosis had no effect upon plasma drug level. In those cases where acidosis developed during the period of anoxia the change in plasma drug concentration was consistent with the change in blood pH. Only in instances of terminal asphyxia with extreme anoxia and marked acidosis was the plasma level so elevated as to suggest an anoxic effect superimposed upon the acidotic rise in drug level.

SUMMARY

1. The concentration of quinacrine and of chloroquine in blood plasma and in erythrocytes was doubled during the administration of 10 per cent CO_2 in O_2 over a 2-hour period. The procedure induced an acidosis corresponding to a drop in blood pH from 7.4 to 6.9.

2. The concentration of drug in muscle tissue and in leucocytes remained unchanged within the errors of measurement. Elevation of plasma drug level in the presence of relatively unchanged tissue concentration indicates that acidosis alters the partition of drug between tissues and plasma.

3. Anoxia of moderate degree did not of itself change the concentration of drug in plasma or erythrocytes. Any small changes encountered were consistent with associated acidosis. In severe asphyxia the combination of extreme anoxia and marked acidosis did give rise to exceptionally high elevation of drug level in plasma and in erythrocytes.

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CHEMOTHERAPEUTIC STUDIES OF 4-AMINO-4'-PROPYLAMINODIPHENYLSULFONE ALONE AND IN COMBINATION WITH SULFADIAZINE IN EXPERIMENTAL PNEUMOCOCCUS INFECTION

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Recently it has been reported that 4-amino-4'-propylaminodiphenylsulfone, a derivative of 4,4'-diaminodiphenylsulfone obtained by alkylation of one amino group, exerts a moderate but well defined chemotherapeutic effect in tuberculosis in guinea pigs (1, 2). This compound is much better tolerated in experimental animals and is as effective as promin (sodium p,p'-diaminodiphenylsulfone N,N'-didextrose sulfonate), one of the more effective sulfone derivatives in experimental guinea pig tuberculosis (3). It has further been shown that in combination with streptomycin the aminopropyl derivative shows markedly more chemotherapeutic effect in tuberculosis than is exhibited either by streptomycin or by the drug alone (4). These observations indicate a need for further information concerning the general chemotherapeutic effectiveness of this drug and chemically related compounds in acute bacterial infections.

Using acute pneumococcus I infection in mice as a test object the activity of 4-amino-4'-propylaminodiphenylsulfone has been tested in the following experiments in comparison with that of 4,4'-diaminodiphenylsulfone and sulfadiazine.

Increasingly favorable results reported with the use of combinations of different chemotherapeutic agents led us also to examine the effect upon pneumococcus infection of the aminopropyl derivative in combination with sulfadiazine. Renal lesions produced by large quantities of sulfadiazine (5-7) render desirable its use in small doses in combination with other antibacterial drugs or antibiotics if it can be shown that such combined treatment is chemotherapeutically effective. Some clinical success has been achieved with combinations of sulfadiazine and penicillin in pneumococcal pneumonia (8, 9), but it has been reported that only a slightly increased therapeutic effect can be demonstrated with sulfadiazine and penicillin in experimental pneumococcus I infection in mice (10). Although the sulfones and sulfonamides may be expected to have a somewhat similar mechanism of action in view of the partial similarity in their chemical structure since their activity is probably associated with the free amino radicals, yet the rather clear demonstration that many sulfones exert a specific chemotherapeutic effect in tuberculosis not demonstrable with sulfonamides (2) indicates the possible existence of a differentiation of mechanism. This may render combined treatment with the two drugs more effective than treatment with either alone.

TOXICITY. Tests for acute toxicity in mice showed that the compound 4-amino-4'-propylaminodiphenylsulfone is tolerated in large doses both by oral and by subcutaneous administration (table 1). All mice survived when given

single doses of 10 grams per kilo, or repeated doses of 15 mg. per mouse given twice daily for four days (totalling 6 gms. per kilo) either by oral administration in 10% gum acacia or by subcutaneous administration in olive oil. The great difference in the tolerated doses between the aminopropyl derivative and the

TABLE 1
Acute toxicity in mice

DRUG	DOSE		ROUTE	NUMBER OF MICE	SYMPTOMS	NUMBER DEAD
	Single	Repeated (B.D.X 4 days)				
4-amino-4'-propyl-amino-diphenyl-sulfone	<i>gms./kilo</i>	<i>gms./kilo</i>				
	10.0		oral	10	Toxic symptoms (1 mouse)	0
	4.0		oral	10	none	0
		0.75 (total 6.0)	oral	3	none	0
		0.5 (total 4.0)	oral	3	none	0
	10.0		subcut.	10	none	0
	4.0		"	10	none	0
		0.75 (total 6.0)	"	3	none	0
4,4'-diamino di-phenylsulfone		0.50 (total 4.0)	"	3	none	0
	0.800		oral	10	Toxic symptoms in all mice	6
	0.500		oral	10	" "	6
	0.250		oral	13	" "	8
	0.150		oral	10	Toxic symptoms (5 mice)	0
	0.125		oral	10	none	0
	4.000		subcut	10	Toxic symptoms in all mice	7
	3.000		"	10	" "	8
	2.000		"	10	" "	4
	1.000		"	10	Toxic symptoms (5 mice)	3
	0.5		"	10	Toxic symptoms (8 mice)	5
	0.250		"	10	none	0
	0.150		"	10	none	0
	0.125		"	10	none	0

parent substance, 4,4'-diaminodiphenylsulfone, of which one-fortieth the dose by weight is toxic by the oral route (0.25 gms. per kilo)¹ and one-twentieth

¹ The sample of 4,4'-diaminodiphenylsulfone used in these experiments (prepared by Abbott Laboratories) did not cause 100% mortality in large doses, but violent symptoms were present in all animals following oral doses of 0.25 gms. per kilo, or subcutaneous doses of 0.5 gms. per kilo, or more. These results are not wholly in accord with those of other authors (11, 12) who found 100% mortality with the higher doses.

the dose by the subcutaneous route (0.5 gms. per kilo) suggested that there is delayed or incomplete absorption of the aminopropyl compound which results in blood concentrations not high enough to cause death. Studies of chronic toxic effects on prolonged treatment with this drug have not yet been made, and are certainly essential to its further development.

ABSORPTION. The method for determination of 4-amino-4'-propylaminodiphenylsulfone in the blood was adapted from the Marshall and Cutting procedure (13) for para-aminobenzenesulfonamide. Values were computed from standard light absorption-concentration curves obtained by the Fisher photo-electric colorimeter upon diazotized and coupled samples of pure 4-amino-4'-propylaminodiphenylsulfone. The sulfone derivative is very sparingly soluble in water and alcohol precipitation of blood protein was accordingly employed. The pale colors of the dye formed on diazotization in alcohol are of disadvantage in rendering results only approximate when blood levels are low because of the low readings obtained on the photometer scale; but this is offset by the nearly complete recoveries when added to blood. Recovery studies upon addition to samples of pooled mouse blood of amounts equivalent to the drug present at 5, 10 and 15 mg. per cent blood level, yielded recoveries of 85 to 98% (table 2). Values were usually reproducible within 3-10% with an occasional maximum variation of 15%.

Blood levels were determined following single doses of the aminopropyl sulfone derivative administered orally in 10% gum acacia and subcutaneously in olive oil. Pooled blood samples from 10 to 14 mice weighing 18-22 grams were obtained by decapitation at 1, 4, 24, and 48 hours following drug administration².

a

Results (table 2) show that the sparingly soluble aminopropyl sulfone derivative is only moderately well absorbed at doses as high as 1 gram per kilo. The highest values were obtained by oral administration of 1 gm. per kilo (20 mg. per mouse) giving blood levels of 6.25-6.8 mg. per cent at 1 hour. Very large doses, 2-4 gms. per kilo, did not increase the blood levels significantly (5.0-6.9 mg. per cent) at 1 hour, indicating a limited absorption of the drug. Following oral administration the drug appeared to have been totally eliminated from the blood within 48 hours with maximum values occurring in one hour. Somewhat lower levels were obtained following subcutaneous administration; possibly it was somewhat more slowly absorbed by this route. Small quantities or traces (2.5 to 0.6 mg. per cent) were still present at 48 hours, indicating a longer retention.

Tests performed by Feinstone (11) on the parent compound, 4,4'-diaminodiphenylsulfone, showed somewhat higher blood levels (7.1-9.1 mg. per cent) to be obtainable in mice by administration of 4-10 mg. per mouse of the drug, doses which fall within the toxic range. Subtoxic doses of 4,4'-diaminodiphenylsulfone (2.5 mg. per mouse) gave lower blood levels, 5.2 mg. per cent (11). Peak blood levels of 4-amino-4'-propylaminodiphenylsulfone comparable in milligrams per cent to the toxic levels of 4,4'-diaminodiphenylsulfone were not obtainable by us even with very large doses, 20 to 80 mg. per mouse (2 to 4 gms. per kilo). On a molar basis 1 gram of diaminodiphenylsulfone represents 1.169

² The possibility of regurgitation was excluded by analyzing the pooled blood of 14 mice decapitated immediately after the administration of the drug. None of the drug was found in the blood.

grams of the aminopropyl derivative, and at equal blood concentrations in milligrams per cent there is present in molecular equivalents approximately 15% less of the aminopropyl compound than of 4,4'-diaminodiphenylsulfone. The aminopropyl compound was therefore not sufficiently absorbed to give blood levels comparable on a molar basis to those obtained with toxic doses of diaminodiphenylsulfone. There is no definite evidence therefore that a real reduction in acute toxicity has been obtained by the substitution of the aminopropyl group for one of the free amino groups of diaminodiphenylsulfone. It is possible that a sharp threshold of tolerance for the sulfone molecule exists in blood and tissues

TABLE 2

4-Amino-4'-propylaminodiphenylsulfone

A. Recovery upon addition to pooled mouse blood (precipitation at 1-10 dilution of blood)

ADDED*	RECOVERED	PER CENT RECOVERY
mg. %	mg. %	
5	4.8	96
10	8.5	85
15	14.7	98

B. Absorption in mice

EXP.	DOSE	BLOOD LEVELS—MG. PER CENT							
		Oral administration				Subcutaneous administration			
		1 hr.	4 hrs.	24 hrs.	48 hrs.	1 hr.	4 hrs.	24 hrs.	48 hrs.
	gm./kilo								
1	1	6.25	5.75	3.73	0	(2.75)	5.03	3.8	(2.5)
2	1	6.80	5.50	(2.60)	0	3.60	3.50	(1.8)	(0.6)
3	2	5.00							
4	4	6.90							

* Quantities equivalent to the drug present at blood levels of 5, 10, and 15 mg. % were dissolved in 1 cc. of 50% acetone, added to 5.0 cc. of blood, and diluted with alcohol to 50 cc. volume.

† Determinations under 3.0 mg. per cent (bracketed figures) are approximate values only, due to the low range of photometer readings.

and that this threshold can be exceeded by the greater absorption of diaminodiphenylsulfone but not by the aminopropyl derivative.

COMPARATIVE THERAPEUTIC RESULTS IN PNEUMOCOCCUS I INFECTION. White female mice weighing 18 to 22 grams and maintained on a diet of purina dog chow were infected with a Type I pneumococcus culture (N.I.H. strain) of such virulence that 0.5 cc. of a 10^{-8} dilution of a six hour broth culture regularly produces fatal pneumococcus septicemia. For the test infection intraperitoneal injection was made of 0.5 cc. of a 10^{-8} dilution of the culture, cultivated in 0.1% dextrose veal infusion blood broth and prepared by serial ten-fold dilution in 1% neopeptone solution. Drugs were administered orally in 10% aqueous gum acacia suspension or subcutaneously in 0.1 cc. of olive or peanut oil. Therapy was begun immediately following infection; and the total dosage, calculated on the basis of grams per kilo per mouse (average weight 20.0 grams), was given over a period of four days (in 8 divided doses, twice daily).

Results of therapeutic tests against type I pneumococcus infection (table 3) show that large doses 2-4 gms. per kilo of the amino propyl sulfone given orally are well tolerated and afford considerable protection, 39-40% of animals sur-

TABLE 3

Therapeutic effectiveness in pneumococcus type I infection in mice

Intraperitoneal injection of 0.5 cc. of a 10^{-6} dilution of 6 hours broth culture. Drug therapy, total grams per kilo per mouse given in divided doses twice daily over 4 days, the initial treatment following immediately after infection.

DRUG	THERAPY		NO. OF MICE	DEATH IN DAYS									SURVIVAL
	Dose	Route		1	2	3	4	5	6	7	8-15		
	gm./kilo											per cent	
4-4'-Diaminodiphenyl-sulfone	0.4	Oral	51	0	1	0	2	22	2	1	2	41.2	
	0.6	"	62	0	2	0	0	6	1	3	7	69.4	
	1.0	"	13	0	1	0	0	0	0	0	0	92.3*	
	0.5	Subcut.	45	0	0	1	2	0	5	8	7	48.9	
	1.0	"	45	0	2	3	0	1	0	0	0	86.7*	
	2.0	"	15	0	2	4	2	0	0	0	0	46.7*	
4-Amino-4'-n-propyl-aminodiphenylsulfone	0.5	Oral	29	0	6	6	2	0	8	2	1	13.8	
	1.0	"	15	0	1	4	2	0	2	2	2	13.3	
	2.0	"	31	0	0	3	1	0	2	6	9	38.7	
	4.0	"	30	0	1	0	1	3	2	5	6	40.0	
	6.0	"	13	0	0	0	1	7	1	1	2	7.7	
	0.05	Subcut.	52	0	23	14	1	0	4	0	3	13.5	
	0.5	"	70	0	13	11	1	1	5	3	15	30.0	
	1.0	"	71	0	1	7	0	0	10	7	12	47.9	
	2.0	"	45	0	0	2	0	0	1	4	8	66.7	
	4.0	"	30	0	0	1	5	2	0	0	4	60.0	
	6.0	"	15	0	0	0	1	5	1	0	4	26.7*	
Sulfadiazine	0.5	Oral	44	0	8	12	3	0	3	5	9	9.1	
	1.0	"	51	0	2	6	0	2	11	6	7	33.3	
	2.0	"	82	0	0	2	3	2	4	8	11	63.4	
	4.0	"	71	1	0	2	1	1	5	4	6	71.8	
	6.0	"	15	0	0	0	0	0	3	1	2	60.0	
	8.0	"	26	0	0	0	1	0	1	0	4	76.9	
	0.5	Subcut.	20	0	4	8	0	0	0	1	6	5.0	
	2.0	"	27	0	0	4	0	1	4	1	3	51.9	
	4.0	"	73	0	0	1	0	0	8	4	4	76.7	
	6.0	"	25	0	0	0	0	0	5	0	3	68.0	
Controls—Infection only	—	—	405	47	350	5	2	—	—	—	—	0.2	

* Toxic symptoms presumably due to drug.

living. Subcutaneously the drug is more effective, 1 to 2 gms. per kilo giving a survival of 48-67%. The protection is not as great as the maximum protection obtainable with the parent sulfone in smaller quantities, 92% surviving with a dosage of 1.0 gm. per kilo given orally, and 87% surviving with the same dose

given subcutaneously. These doses of the parent sulfone, however, are associated with some symptoms of drug toxicity in infected animals and are therefore beyond the safety limits.

A comparison of the approximate therapeutic indices (LD_{50}/CD_{50})³ of the two sulfones shows that, when subcutaneous treatment is used the CD_{50} of the aminopropyl derivative is approximately 1.0 gm. per kilo with no acute toxic dose⁴, or a therapeutic index of more than 10, as compared with diaminodiphenylsulfone the LD_{50} of which is about 0.5 gms. per kilo, giving a therapeutic index of approximately 1.0. By oral treatment fifty per cent survival was not obtained even with large doses (4.0–6.0 grams per kilo) of the aminopropyl compound. In contrast fifty per cent survival could be obtained with diaminodiphenylsulfone in doses of 0.6 grams per kilo or less but this quantity is clearly toxic when given in single doses. It should be pointed out that the comparison on the basis of a therapeutic index is made for convenience only in showing the relation of the tolerated single dose to the total effective therapeutic dose weight for weight. It does not take into consideration the absorption, relative effective blood levels, or retention of the substances concerned. No quantitative comparison of the antibacterial power of the two drugs, on a molecular basis and in relation to the levels absorbed into the blood, can be made without further dietary experiments. The results indicate, nevertheless, that the aminopropyl sulfone derivative retains considerable therapeutic efficacy in pneumococcus I infection despite its poor absorbability. It is tolerated in much larger doses by weight than the parent sulfone, which may however be accounted for by the fact that it is less readily or completely absorbed.

A comparison of the activity of the aminopropyl sulfone with that of sulfadiazine against pneumococcus infection shows that in doses of 2 to 4 gms. per kilo by subcutaneous injection the sulfone derivative is approximately as effective as sulfadiazine. Higher doses (6.0 gms. per kilo divided into 8 doses over 4 days) of sulfadiazine were tolerated and maintained their protective action, but the sulfone derivative in similarly large doses, while showing no symptoms of acute toxicity in normal animals, exhibited toxic symptoms in infected animals and gave a low percentage of survival. This increased toxicity of sulfones in infected as compared with normal animals has been reported before (12). By oral administration sulfadiazine is definitely more effective than the sulfone derivative, and is also better tolerated in large doses in infected animals.

It is of interest that subcutaneous administration of the aminopropyl compound in oil gave better protection than oral administration in gum acacia. A comparison of the blood levels by both routes in table 2 shows that the better therapeutic results obtained by subcutaneous administration are apparently not due to higher blood levels. It is possible that subcutaneous injection in oil

³ Used in these studies as the ratio of the single dose by weight which gives approximately 50% mortality in normal mice and the total curative dose which gives approximately 50% survival in infected mice.

⁴ No deaths with doses of 10 gms. per kilo, a quantity probably beyond the threshold of absorption.

forms a reservoir of the drug from which it is mobilized slowly, maintaining an effectively bacteriostatic concentration of the drug in the blood and tissues over a longer period. Effective therapy appears to depend therefore, not on obtaining high peak levels of the sulfone in the blood stream but rather on its maintenance at low levels over an extended period.

COMBINED SULFADIAZINE AND SULFONE THERAPY IN PNEUMOCOCCUS INFECTION IN MICE. The effect of combined therapy consisting of sulfadiazine administered orally and 4-amino-4'-propylaminodiphenylsulfone administered subcutaneously was tested in four experiments using the drugs at levels of 0.25, 0.5

TABLE 4

Combined therapy in pneumococcus I infection in mice

Effect of sulfadiazine (oral administration) and 4-amino-4'-propylaminodiphenylsulfone (subcutaneous administration). Drug therapy: Total grams per kilo per mouse given in divided doses twice daily over 4 days, the initial treatment following immediately after infection.

EXP.	SULFADIAZINE (ORAL)	4-AMINO-4'-PROPYLAMINODIPHENYLSULFONE. (SUBCUTANEOUS)	NUMBER OF MICE	SURVIVAL (14 DAYS)
	<i>gm. per kilo</i>	<i>gm. per kilo</i>		<i>per cent</i>
I	1.0	0	21	57
	0	1.0	26	54
	1.0	1.0	25	88
II	0.5	0	25	8
	0	0.5	25	24
	0.5	0.5	25	68
III	0.25	0	27	0
	0	0.25	27	11
	0.25	0.25	27	56
IV	0.25	0	24	0
	0	0.25	25	16
	0.25	0.25	24	38

and 1.0 grams per kilo, doses previously determined to give approximately fifty per cent or less survival when used alone. In all of the four experiments the per cent survival using both drugs was considerably higher than with the same amount of either drug alone (table 4). At levels having little or no therapeutic value with either drug alone (Exp. 2, 3, 4), the combined therapy resulted in a definitely greater percentage of surviving mice than was accounted for by the sum of the effects of the two drugs used alone. This indicates the possible existence at the lower dosage levels of a potentiation of action, in the sense that the combined action is greater than the algebraic sum of effects of the individual components. At higher dosage levels (of 1.0 gm./kg. giving approximately 50% survival with the individual drugs) this effect did not appear; 100 per cent survival could not be obtained either by combined therapy or with large doses of the individual drugs.

The term potentiation is used here in the pharmacological sense as it has been defined by Goodman and Gilman (14) and by Sollmann (15). The term synergy has often been employed with the same meaning in experiments measuring the action *in vivo* of combinations of antibacterial substances. In such *in vivo* experiments many variables such as animal resistance, number of organisms, drug-resistance of the strain, etc., may be determining factors. Under these conditions the terms synergy or potentiation should be used in a limited sense only, for assessment of effective methods of combined therapy. They should not be interpreted as proof of a qualitative change in mechanism of chemotherapeutic action due to the combined therapy.

TABLE 5

Combined therapy in pneumococcus I infection in mice

Effect of sulfadiazine and 4-amino-4'-propylaminodiphenylsulfone, both by subcutaneous administration.

EXP.	SULFADIAZINE (SUBCUTANEOUS)	4-AMINO-4'-PROPYLAMINODIPHENYLSULFONE (SUBCUTANEOUS)	NUMBER OF MICE	SURVIVAL
	!gm. per kilo	gm. per kilo		per cent
I	0.5	0	20	30
	0	0.5	20	55
	0.5	0.5	20	95
	0.25	0	20	15
	0	0.25	19	21
	0.25	0.25	20	75
II	0.5	0	20	50
	0	0.5	20	45
	0.5	0.5	20	70
	0.25	0	20	5
	0	0.25	20	15
	0.25	0.25	20	55

It was felt that a comparison of the effect of combined therapy with that of larger doses of either drug alone would be of help in determining whether the complementary action of the two drugs represents simply an effect of increased drug dosage, or whether the combination of the two drugs acts in a qualitatively different and more effective manner in eliminating the infection.⁵ A comparison between separate experiments (table 4) of percentage survival using combined therapy with survival using double the dose of either drug alone, shows somewhat greater survival with combined therapy, [11 (68-57), 32 (56-24) and 14 (38-11) per cent] than with twice the dose alone (Exps. I, II and III, respectively). These differences, though occurring consistently, were not sufficiently great to be of definite significance. This is particularly true in view of the different

⁵ This comparison is used as a rough approximation only in view of the fact that it is based on dosage by weight, and only large differences in survival can be considered significant. Quantitative methods are not available for this type of study.

routes of drug administration used, and of the relatively large variations normally appearing between individual chemotherapeutic experiments of this kind.

A comparison within a single experiment of the combined therapy (0.25 gms. per kilo of each drug) with the effect of the individual drugs at the same dosage (0.25 gms. per kilo), and at double the dose (0.5 gms. per kilo), was next made (table 5). The results again show a greater effect of combined therapy in small doses (0.25 gms. per kilo of each drug), the percentage survival being greater than the sum of effects from the same dosage of each drug given individually. They also show that the potentiation, in the above sense, is not always associated with a therapeutic action significantly greater (20 and 5 per cent greater in experiments I and II, respectively) than the action of double the dose of either drug alone. In this type of experiment the demonstration of a potentiation does not mean, therefore, that combined therapy with two drugs provides a qualitative change in chemotherapeutic action exceeding that of larger doses of either drug alone. It is of value, however, from the standpoint of establishing a basis for effective therapy under conditions where a limited quantity of a particular drug should be employed.

DISCUSSION. The complementary action demonstrated here between 4-amino-4'-propylaminodiphenyl sulfone and sulfadiazine appears to be more pronounced than the increased therapeutic effect obtained in pneumococcus type I infection in mice in response to simultaneous treatment with penicillin and sulfadiazine, which is reported to fall far short of the sum of the individual effects of the drugs (10). More work is needed with standardized conditions of infection, multiple strains of pneumococci and larger series of animals, before general conclusions as to the relative values of combinations of sulfones or penicillin with sulfadiazine may be reached. There appears to be no such striking complementary effect in this work as has been reported from combinations of sulfadiazine and streptomycin in mice infected with certain strains of *H. influenzae* where the combined therapy resulted in 70% more survival than that represented by the summation of percentage survival obtained with the two substances used individually (16), or between combinations of sulfapyridine and penicillin in *B. hemolytic* streptococcus infection in mice where 80% more survival was obtained with combined therapy than from the summation of per cent survival with individual substances (17). In our experiments the maximum increase in effect with combined therapy resulted in 45% more survival than that of the sum of the individual effects of the drugs. It does appear however that sulfone drugs exert a definite moderate complementary effect in combination with other therapeutic substances, not only with sulfadiazine in pneumococcus I infection in mice as demonstrated in this study, but also with streptomycin in tuberculosis of guinea pigs (4, 18).

From the standpoint of effective treatment of pneumococcus type I infection in mice, it is clear that when small doses of sulfadiazine are employed the supplementary use of 4-amino-4'-propylaminodiphenylsulfone is of advantage. Further work, particularly concerning both the acute and chronic toxic effects of the aminopropyl sulfone is necessary. Further investigation of the possibility

of still better complementary action between the sulfones and the antibiotics such as streptomycin are also indicated before any general application of these results to therapy in pneumococcus infection is to be considered.

SUMMARY

The toxicity and chemotherapeutic activity of 4-amino-4'-n-propylamino-diphenylsulfone have been studied in experimental pneumococcus I infection in mice in comparison with the parent substance, 4,4'-diaminodiphenylsulfone, and with sulfadiazine.

The n-propyl derivative retains a moderate chemotherapeutic activity, not as great however as that of 4,4'-diaminodiphenylsulfone. The aminopropyl derivative is tolerated in normal animals in much larger doses, weight for weight, than the parent sulfone, thus giving it a better chemotherapeutic index; and this appears to be accounted for wholly or in part by the fact that it is not as well absorbed as the parent compound. In very large doses, divided over several days, the aminopropyl compound appears to be more toxic for infected mice than for normal mice. It is not as effective as sulfadiazine on oral administration, and is less well tolerated in large doses in infected animals.

The simultaneous administration of small doses of sulfadiazine and the n-propyl derivative gave a degree of chemotherapeutic effectiveness greater than the sum of effects from the individual components.

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SOME EFFECTS OF STILBESTROL AND ITS MONOMETHYL ETHER IN THE IMMATURE MALE RAT¹

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The effectiveness of certain of the estrogens in stimulating growth of the male seminal vesicles has been reported by several observers (1-3). Two modes of action were postulated as a result of these studies. One, applicable to the lower dose ranges, interpreted the result as due to pituitary stimulation resulting in increased testicular function. The second mechanism, applicable to the higher dosages and the results found in castrated males, was that of direct stimulation.

We were interested in assaying stilbestrol for its potency in seminal vesicle stimulation and also in determining the relative effectiveness of the monomethyl ether derivative³ by the same criterion. We had been able to show previously that this minor change in the molecule had induced marked changes in the pharmacology of the compound (4, 5).

METHODS AND OBSERVATIONS. Immature male rats three weeks old and weighing about 30 grams were injected subcutaneously with olive oil, stilbestrol, or its monomethyl ether in oil in varying doses twice daily for five days. The testes and seminal vesicles were removed and weighed on the morning of the sixth day. (The data relevant to the testes are shown in table I but are not discussed since the apparent differences cannot be considered statistically valid.) Some of the tissues were fixed in Bouin's solution for later examination. The animals used were of the Sherman strain and were selected with careful regard to weight and age. This was considered essential in the study of seminal vesicle changes in the young rat since between four and six weeks of age normal growth changes in their accessory organs are so marked that the use of a heterogeneous group of animals could easily have masked the results.

From the results (table I and fig. 1) it is clear that stilbestrol in daily injections was the more effective in bringing about seminal vesicle growth in total doses of 0.5 gamma to 100 gamma. Its ether on the other hand, caused a slight but statistically significant increase in seminal vesicle weight at 1 gamma and a larger gain at dosage levels of 8 gamma and 100 gamma but did not appear to stimulate the growth of the seminal vesicles at 2 gamma. Examination of table I shows that the increase in weight which followed the injection of 1 gamma of the monomethyl ether was the least significant of any deviation from the normal noted.

Another difference between the two estrogenic substances tested appeared in

¹Supported in part by a grant from the Wallace & Tiernan Co.

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³The trade name for the monomethyl ether is monomestrol.

a comparison of their respective slopes when the weight changes are plotted against the dosage (fig. 1). Their slopes differ from each other in that the points for the daily injection series of stilbestrol fall rather well along a straight line from dosage 0.5 gamma to 8 gamma at which concentration the maximum response within the limits of this experiment has been obtained. The ether, however, begins to consistently stimulate seminal vesicle growth somewhere between 2 gamma and 8 gamma and is still increasingly effective with increase in dosage at the 100 gamma level.

Cytological observations of the seminal vesicles of animals which received eight gamma or more of either estrogenic substance showed marked metaplasia

TABLE I

3 week old male rats injected with stilbestrol or its monomethyl ether in oil for 5 days, twice daily. Each injection consisted of 0.1 cc. of solution

SUBSTANCE INJECTED	DOSE	NO. ANIMALS	AVERAGE SEMINAL VESICLE WEIGHT	AVERAGE TESTICULAR WEIGHT
	γ		mg.	mg.
Oil (controls)		50	5.6	185.7
Stilbestrol	0.5	27	7.0*	—
	1.0	31	7.3*	—
	2.0	11	12.2	121
	8.0	5	24.0	247
	25.0	6	22.3	158
	100.0	6	21.1	108
Monomestrol	.5	17	5.5	—
	1.0	22	6.4†	—
	2.0	11	5.2	195
	8.0	6	12.4	374
	25.0	6	15.9	111
	100.0	6	18.8	101.

* The probability is less than .01 that this figure is the same as the control group.

† The probability is less than .05 but more than .01 that this figure is the same as the control group.

of the fibromusculature. That is, the number of cells in the layer of connective tissue and muscle just under the lining epithelium was grossly increased.

SINGLE INJECTION SERIES. It seemed that more information on the pharmacology of the monomethyl ether of stilbestrol would be forthcoming if a comparison of the effects of single versus repeated injections were made. Accordingly immature male rats (three weeks old) were injected once with 2 gamma to 200 gamma of the ether or stilbestrol. At all the dosage levels tested stilbestrol given in a single injection was less effective than when the same amount was divided and given in ten injections, (tables I and II, fig. 1). In contrast there was little if any difference in the effectiveness of the ether when given once or over a five day period (tables I and II, Fig. 1). Furthermore, when as much as

100 gamma of the ether were given, doubling the dose produced no further increase in size of the seminal vesicles. Injection of 8 gamma to 200 gamma of the ether resulted in larger seminal vesicles than did injections of the same amount of stilbestrol.

To test the hypothesis that the effect was due to the direct action of the estrogens on the seminal vesicles two groups of five males each were injected immediately following castration with 200 gamma of stilbestrol or the same amount

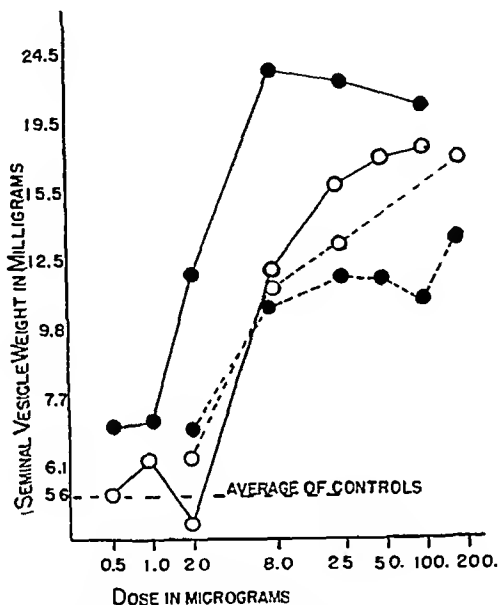


FIG. 1. THE RELATIONSHIP BETWEEN THE RESPONSE OF THE SEMINAL VESICLES OF THE IMMATURE MALE RAT TO SINGLE AND DIVIDED DOSES OF STILBESTROL, AND ITS MONOMETHYL ETHER

Injections were made twice daily for 5 days in the repeated injection series. In both instances the animals were killed 6 days after the first injection.

The figure shows the relation, on a logarithmic grid, between the dosage of estrogen and the weight of the seminal vesicles in the immature male rat. The curves for the repeated injection series are drawn as solid lines, the single injection series as interrupted lines. The curves containing the solid circles refer to diethyl stilbestrol, the open circles to the monomethyl ether of stilbestrol.

of its derivative. As in the other singly injected animals the seminal vesicles were weighed on the morning of the sixth day and were found to give results not significantly different from normal males, similarly injected.

DISCUSSION. The results of the repeated injection series indicated that the injection of as much as 8 gamma or more up to the maximum amount given, namely 100 gamma, was followed by a marked enlargement of the seminal vesicles. This was very probably due to the direct action of estrogenic substances on the seminal vesicles. That this was the case is supported by the fact

that the castrated animals which received in one injection 200 gamma of stilbestrol or its ether showed an enlargement of their seminal vesicles comparable to those of the intact animal similarly injected. It has long been known that large doses of other estrogens have brought about an increase in the weight of the seminal vesicles (2).

Further, the fact that the increase in weight was accompanied by a gross increase in the thickness of the fibromusculature was a result which has also been associated with the direct action of estrogens on the seminal vesicle (2, 3).

Daily injections of small doses of stilbestrol (0.5 gamma and 1 gamma) were also slightly stimulating and appeared to have brought about the seminal vesicle growth directly since the stimulation seemed to be continuous and consistently increasing with dosage.

TABLE II

3 week old male rats injected once with stilbestrol or monomestrol. Organs weighed 6 days later. Each injection consisted of 0.1 cc. of solution

TREATMENT	DOSE	NO. ANIMALS	AVERAGE SEMINAL VESICLE WEIGHT	AVERAGE TESTICULAR WEIGHT
	γ		mg.	mg.
Stilbestrol	2	6	7.0*	175
	8	4	10.2	414
	25	5	11.9	416
	50	6	11.8	242
	100	4	10.5	211
	200	12	13.9	388
Monomestrol	2	8	6.5	206
	8	5	11.7	300
	25	6	13.3	189
	50	6	16.9	154
	100	4	18.3	166
	200	10	17.5	174

* The probability is less than .01 that this figure is the same as the control group.

The results of the injection of small amounts of the ether, on the other hand, were such that two interpretations were possible. Since the injection of a total of 0.5 gamma or 2 gamma did not bring about an increase in the seminal vesicle weight while 1 gamma did cause a slight increase it could be said that at the 1 gamma level the pituitary was stimulated. The stimulation of the pituitary in turn brought about a greater androgen secretion by the testes which resulted in an enlargement of the seminal vesicles. The fact that at the two gamma level the seminal vesicles were not increased in size but were even smaller than the average for the controls might again have been due to the fact that at such a concentration inhibition of the pituitary had occurred. This explanation is reasonable according to our current hypothesis concerning pituitary function (6). However, since the significance of the seminal vesicle increase in weight at one gamma

was somewhat questionable, the following interpretation was considered more conservative. That is, the monomethyl ether was ineffective in stimulating any measurable growth of the seminal vesicle until 8 gamma had been injected and then its action was direct on the end organ and not by way of the pituitary.

The fact that the ether was the less effective in the lower dose range may have been the result, at least in part, of too slow a release of stilbestrol from the monomethyl molecule to produce a stimulating dose of stilbestrol at any one time. That such a conversion does take place has been demonstrated (7).

Certain differences in the pharmacology of the two estrogenic substances tested were noted in a comparison of their effectiveness when given once rather than in divided doses. The fact that the monomethyl ether was the more effective when the materials were injected once rather than repeatedly while it was the less effective in stimulating seminal vesicle growth in the daily injection series indicated that the addition of the methyl group while decreasing the immediate estrogenic potency of the compound as previously determined by the Allen-Doisy technique (8), did at the same time prolong the period during which there was an effective estrogenic substance acting on the seminal vesicles. These results in the male seemed especially interesting in that a somewhat similar effect has been reported for female rats, that is, following the injection of the methyl ether vaginal cornification was found to be more than usually prolonged as compared with the effect produced by other estrogens similarly injected (9.)

SUMMARY AND CONCLUSIONS

Study of the seminal vesicles of immature male rats injected twice daily for five days with total doses of 0.5 gamma to 100 gamma stilbestrol or its monomethyl ether showed that at doses as low as 8 gamma in the monomethyl ether series and 0.5 gamma in the stilbestrol series these organs were stimulated. Injections of as much as 8 gamma resulted in marked enlargement of the seminal vesicles but at such dosages the enlargement was the result, at least in large part, of the direct action of the estrogens on the fibromusculature. At every dosage tested stilbestrol in repeated injections caused greater seminal vesicle growth than did the monomethyl ether.

Single injections of 2 gamma to 200 gamma of the two estrogens showed that stilbestrol was consistently less effective when given in one injection than when the same amount was given in divided doses. Such a loss in potency when a single injection was given did not obtain in the case of the monomethyl ether. Animals receiving 8 gamma to 200 gamma of the ether exhibited better seminal vesicle growth than animals receiving equal amounts of stilbestrol. The growth in both instances was shown to be due to the direct action of estrogens on the seminal vesicles. The greater effectiveness of the monomethyl ether was attributed at least in part to its ability to gradually release stilbestrol as well as to any inherent estrogenic potency.

Because of the greater effectiveness of the monomethyl ether as compared to stilbestrol when given in one injection and its lesser effectiveness when given daily no statement as to the relative potency of the two compounds can be made.

In other words the conditions under which these substances were administered determined their relative effectiveness.

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THE PHARMACOLOGY OF COMPOUNDS STRUCTURALLY RELATED TO HYDROXYTYRAMINE

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Konzett (1) has described the pharmacology of a series of N-alkyl homologues of epinephrine in which the methyl group on the nitrogen has been replaced by ethyl, propyl, isopropyl, butyl and isobutyl groups. The N-isopropyl and N-ethyl homologues were found to be potent bronchodilator drugs, being more active than epinephrine. Siegmund, Granger, and Lands (2) have shown these two substances and the N-sec.-butyl homologue to be as effective as epinephrine in preventing histamine asthma in unanesthetized guinea pigs and anaphylactic bronchoconstriction in perfused lungs of guinea pigs previously sensitized to horse serum.

Konzett (3) has shown the N-ethyl and N-isopropyl homologues of epinephrine to be very effective in causing relaxation of smooth muscle usually relaxed by epinephrine. Lands, Nash, McCarthy, Granger and Dertinger (4) have found the action of the N-sec.-butyl homologue to be very similar to that of the above compounds and have suggested that these substances are sympathin I-mimetic agents. In an effort to study this action in more detail, we have investigated the pharmacology of a reasonably complete series of compounds structurally related to hydroxytyramine. This communication describes the results we have obtained.

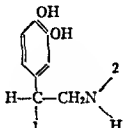
EFFECTS ON BLOOD PRESSURE. Dogs were anesthetized with sodium pentobarbital, atropinized, the common carotid artery cannulated for blood pressure recording and the trachea cannulated for the recording of respiration. All drugs were dissolved in distilled water and injected into the exposed femoral vein. The results obtained are shown in table 1 and fig. 1. Nor-epinephrine ('Arterenol'), a racemic compound, has an l-epinephrine ratio of 1.35, is 1.5 times more pressor than racemic epinephrine, and is the most potent vasopressor drug. The ethane derivatives, 'Epinine' (epinephrine ratio of 10.6), and hydroxytyramine (epinephrine ratio of 50), are distinctly less potent. From the above data, it would appear that the greatest reduction in pressor potency results from the removal of the hydroxyl group from the beta carbon of nor-epinephrine. Nor-epinephrine is 5 times more pressor than 'Epinine.' Using nor-epinephrine as a basis for comparison, it is readily apparent that N-methyl substitution reduces pressor potency and that with the N-ethyl and larger alkyl homologues described here strong depressor effects are obtained. The structure rather than the size of the N-alkyl group appears to be the more important factor determining

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depressor potency. Thus, the *N*-*tert*-butyl and *N*-isopropyl ('Isuprel') homologues are most depressor and the *N*-*n*-butyl homologue is the least depressor. If 'Isuprel' is used as a basis for comparing depressor effects, it will be noted that the removal of the alcoholic hydroxyl from the beta carbon (No. 1554—table 1) leads to a very great reduction in depressor potency and that the conversion of the alcoholic hydroxyl to the corresponding ketone (No. 1590—table 1) produces a compound with weak pressor action. The alcoholic hydroxyl group

TABLE 1

The effect of derivatives of hydroxytyramine on the blood pressure of the dog

<div></div> COMPOUND*	STRUCTURE		EFFECT ON BLOOD PRESSURE				APPROX. EPI RATIO
	1	2	Dose	Number of experi- ments	Change in B P.	Duration	
Hydroxytyramine.. 'Epinine'	H H	H CH ₃	 17.0	16	 +41	 2-3	50.0† 10.6
1554	H	CH(CH ₃) ₂	730 0	13	{ +18‡ -30	3-10	
Nor-epinephrine	OH	H	2 1	14	+39	2-3	1.35
d,l Epinephrine	OH	CH ₃	3 1	6	+49	2-3	2.00
1516	OH	C ₂ H ₅	1 7	13	{ +8 -32	transient 5-23	
'Isuprel'	OH	CH(CH ₃) ₂	1 1	22	-46	3-17	
1505	OH	C(CH ₃) ₃	0 9	6	-43	5-15	
1424	OH	CH(CH ₃)C ₂ H ₅	1.0	7	-33	2-4	
1960	OH	C ₂ H ₅	17 0	22	-45	2-10	
'Kephriene'	=O	CH ₃	130 0	8	+39	3-12	83.0
1590	=O	CH(CH ₃) ₂	1200 0	8	{ +21 -28§	5-8	1400 0
Epinephrine	OH	CH ₃	1 0	16	+25	2-3	

* Solutions prepared from the hydrochloride or acetate (No 1505 and 1424) salts. Optically active compounds used as racemic mixtures

|| Dose given as l-epinephrine base

† Value reported by Alles (1933)

‡ Strong pressor component with a resultant rise followed by a fall in 5 out of the 13 experiments The +18 mm Hg is the average rise obtained in these 5 experiments.

§ Response variable In 5 experiments only rises were obtained

therefore appears to be of great importance for both pressor and depressor stimulation.

EFFECT ON THE HEART. Effect on the heart beat was determined in frogs by perfusion, and in anesthetized dogs by direct myocardiographic (Cushny) recording of the contractions of the left ventricle. With the perfused frog heart, injections were made directly into the perfusion cannula. Depending upon the dose, epinephrine exerts one or the other of two effects. With small doses

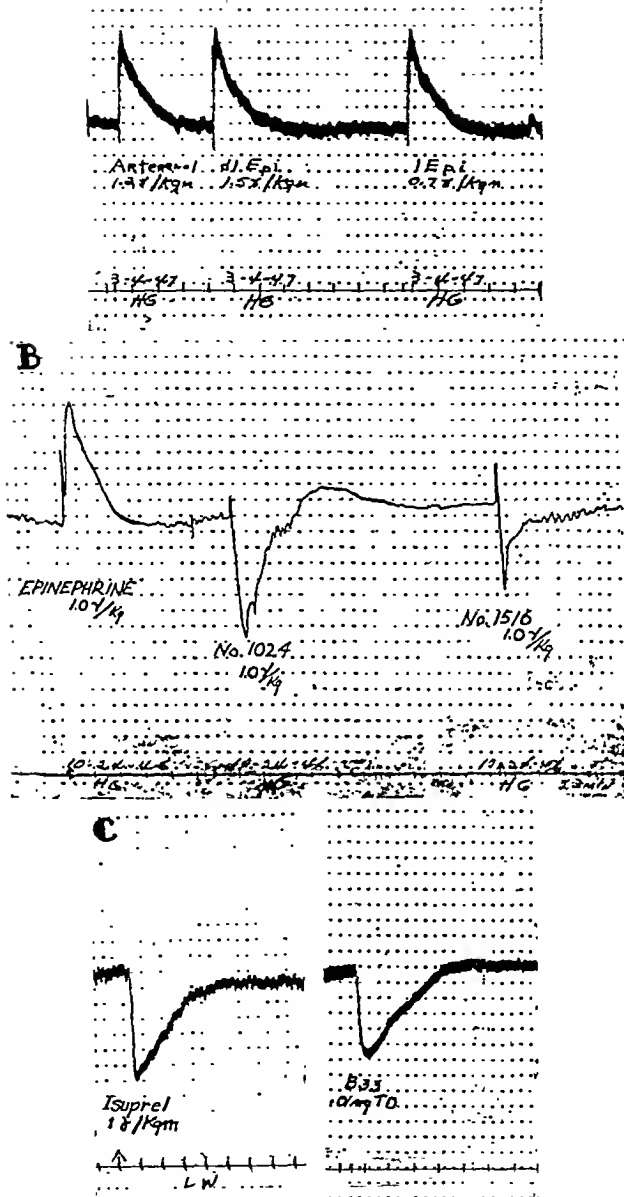


FIG. 1. EFFECT ON CAROTID BLOOD PRESSURE OF ANESTHETIZED DOGS

A. Comparative pressor action of nor-epinephrine ('Arterenol'), racemic epinephrine and epinephrine. Drugs were injected into the exposed femoral vein. The doses were 1.2, 1.5, and 0.7 mg./kg., respectively. Time intervals in minutes.

B. A comparison of the effects on blood pressure of the intravenous injection of 1.0 mg./kg. of epinephrine, 'Isuprel' (No. 1024) and the N-ethyl homologue (No. 1516). Time intervals in minutes.

C. A comparison of the effect on blood pressure of the intravenous injection of 1.0 mg./kg. of 'Isuprel' and the N-sec-butyl homologue (B-33). Time intervals in minutes.

(0.001–0.01 mcg.) one obtains an increase in both rate and amplitude. As the amount of drug injected is increased (0.025–0.10 mcg.) the ventricle stops beating in diastole. These results are in essential agreement with those obtained by Sollmann and Barlow (5). The vasodepressor compounds, such as 'Isuprel' or the *N*-*sec*.-butyl or *N*-*tert*.-butyl homologues, cause only increased amplitude and frequency at these concentrations (fig. 2). Myocardiograms of the left ventricle of anesthetized dogs show that those members of this series that are active vasodilators cause marked stimulation. The presence of hydroxyl groups on the phenyl ring appears to be unnecessary for this effect, inasmuch as the phenyl analogue of 'Isuprel' (1-phenyl-2-isopropyl-aminoethanol) is quite effective in causing cardiac stimulation (fig. 3). In several experiments, pulse rates

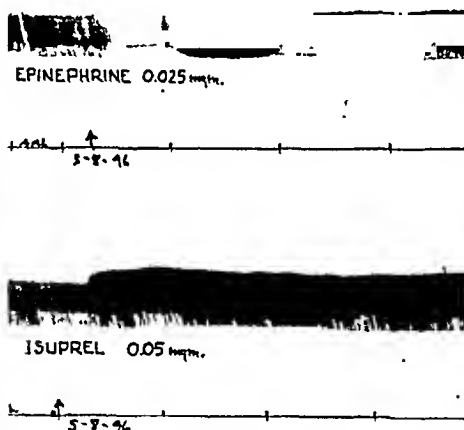


FIG. 2. EFFECT ON THE PERFUSED FROG HEART

The drugs were injected directly into the perfusion cannula. The doses are: epinephrine, 0.025 mg.; 'Isuprel,' 0.05 mg.

and blood pressures were determined in anesthetized non-atropinized dogs following intravenous injection of the drugs. The average changes in pulse rates are shown in table 2. Examination of these data indicates that 'Isuprel' causes the greatest amount of stimulation, being about twice as potent as the *N*-ethyl (No. 1516) and *N*-*tert*.-butyl homologues, and five times more stimulating than epinephrine. The *N*-*n*-butyl homologue has about one-tenth of the stimulating effect of 'Isuprel'. The removal of the alcoholic hydroxyl from the beta carbon (No. 1554) or its replacement by oxygen to give the corresponding ketone (No. 1590) leads to a great reduction in the cardiac stimulating effect. This is in agreement with the finding that these substances have little effect on blood pressure in doses up to 1.0 mg./kg. (table 1). The members of this series that are

most effective in causing vasodepression are also effective in increasing pulse rates. It is improbable that reflex cardiac stimulation contributes much to this effect, inasmuch as marked stimulation of the isolated perfused rabbit heart has been described for 'Isuprel' and No. 1424 by Lands, *et al.* (4).

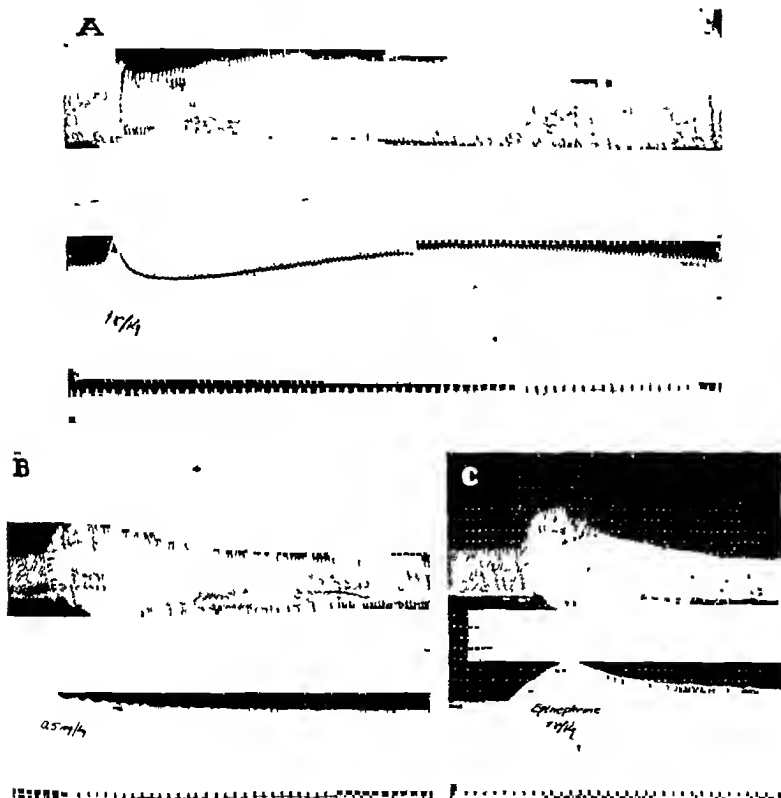


FIG 3 EFFECT ON THE DOG HEART IN SITU

From above downward are myoeardiogram, earotid blood pressure and time in 5 second intervals. All drugs were injected into the exposed femoral vein. A 'Isuprel,' 10 mcg/kg, B 1-phenyl-2 isopropylaminoethanol HCl, 0.5 mg/kg, C epinephrine, 10 mcg/kg.

EFFECT ON THE BRONCHIOLES Excised guinea pig lungs were perfused by the method of Sollmann and von Oettingen, as modified by Thornton (6). All drugs were dissolved in distilled water and injected into the perfusion stream near the lung. Bronchoconstriction was induced by histamine acid phosphate. In all instances, control responses to histamine were obtained and were compared with the responses obtained when the constricting doses of histamine were injected

with the bronchodilating drugs. Results obtained are shown in table 3. 'Epinephrine' and nor-epinephrine are comparatively weak bronchodilating drugs. In the absence of an hydroxyl on the beta carbon of the side chain (Nos. 1554 and 1590) the N-isopropyl group does not appear to increase bronchodilating action. Those compounds containing an hydroxyl on the beta carbon and also an N-alkyl group show marked bronchodilating action. The structure of the N-alkyl group appears to be important for this action inasmuch as both N-*sec*.-butyl and

TABLE 2

The effect of derivatives of hydroxytyramine on heart rate of anesthetized dogs

COMPOUND	DOSE	NUMBER OF EXPERIMENTS	BEFORE INJECTION	MAXIMUM INCREASE AFTER INJECTION	DURATION OF SOME INCREASE IN RATE
	<i>mcg./kg.</i>		<i>beats/min.</i>	<i>beats/min.</i>	<i>min.</i>
Epinephrine..	1	3	136	16	1.5
1516	1	7	149	40	15.0
'Isuprel'	1	6	151	85	17.5
1505	1	3	134	49	20.0
1424	1	3	209	35	19.0
1960	10	4	143	82	5.0
1554	325	4	146	48	8.0
1590	1000	3	141	16	10.0

TABLE 3

Bronchodilator action of derivatives of hydroxytyramine (perfused isolated guinea pig lung)

COMPOUND	AMOUNT INJECTED	RESPONSE TO HISTAMINE	RESPONSE TO HISTAMINE + COMPOUND
	<i>mg</i>	<i>cc./min</i>	<i>cc./min.</i>
'Epinephrine'	0.250	33/14	34/37
1554	1.000	48/25	49/48
Nor-epinephrine	1.400	35/17	34/33
d,l-Epinephrine	0.005	33/15	33/28
1516	0.005	45/23	46/42
'Isuprel'	0.005	33/14	32/36
1505	0.005	42/21	41/42
1424	0.005	38/17	35/39
1960	0.005	42/17	41/36
'Kephrene'	0.050	44/24	43/38
1590	6.400	44/18	44/47
Epinephrine	0.005	37/18	34/32

N-*tert*.-butyl homologues were found to be more active than the N-*n*-butyl homologue. Bronchodilatation appears to be closely correlated with the vaso-depression obtained with this series of derivatives.

EFFECT ON THE INTESTINE AND UTERUS. The small intestine is relaxed by epinephrine in a low dilution, presumably due to its sympathin I-mimetic action. The isolated guinea pig ileum has been used here as the test organ for these relaxing (inhibitory) actions. Isolated ileal segments of recently killed guinea pigs

TABLE 4

Effect on the isolated intestine and uterus of compounds structurally related to hydroxytyramine

COMPOUND*	DILUTION	ACTION ON GUINEA PIG ILEUM	ACTION ON THE UTERUS	
			Guinea pig	Rabbit
	<i>millions</i>			
'Epine'†	10.0	N.A.†	E	E/N.A.
	4.0	I†		
	0.4	E†	E	E
1554	0.4	I		
	0.1	I	N.A.	I/N.A.
Nor-epinephrine	10.0	N.A.	E	E
	4.0	I	E	E
	0.4	E/I†		
d,l-Epinephrine	20.0	I	E	E
	1.0	E	E	E
1516	20.0		E/N.A.	
	10.0	I		E
	1.0	I	E	E
	0.4	E/I		
'Isuprel'	40.0	I	I	I
	1.0	I	I	I
1505	10.0	I/N.A.		I
	1.0	I	I	I
	0.4	I	I	
1424	20.0	I	I	I
	0.4	I		
1960	4.0	N.A.		I/N.A.
	1.0	I	I	I
	0.4	I		
'Kephrine'	1.0	I		
	0.4	I		
	0.1	I	I	E
1590	0.1	I	N.A.	N.A.
Epinephrine	20.0	I		E
	4.0	I/E	E	
	1.0	E		

* The hydrochloride or acetate salts were dissolved in distilled water. Epinephrine dilution represents the amount of base rather than the salt. d,l-Epinephrine hydrochloride dilution represents the amount of the salt.

† I = inhibition; E = excitation; E/I = excitation followed by inhibition; N.A. = no action.

were suspended in a muscle bath of 200-cc. capacity; the temperature was maintained at 37.5°C. The bath was filled and emptied twice between each determination of drug action. Results obtained are shown in table 4 and figure 4.

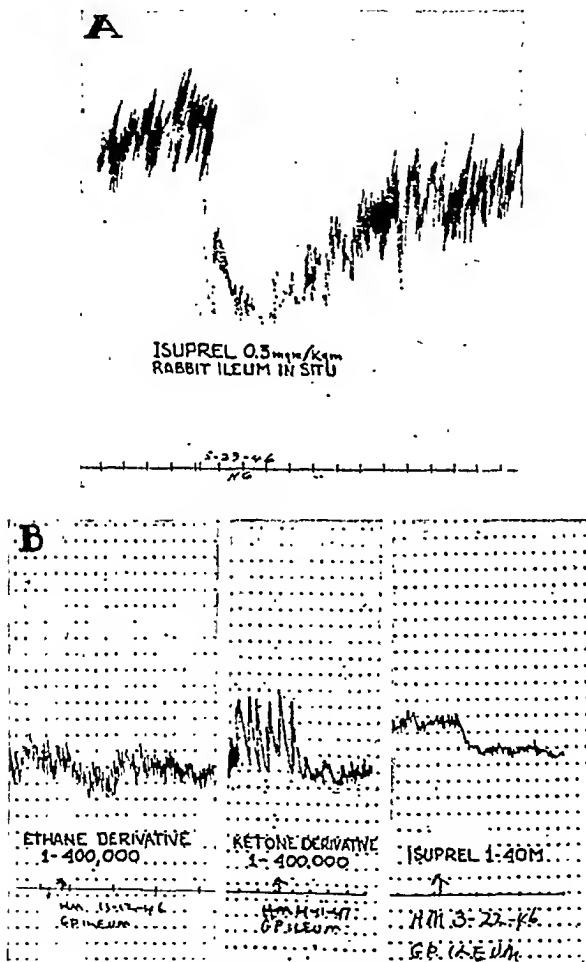


FIG. 4. EFFECT ON THE SMALL INTESTINE

A. Rabbit ileum in situ. 'Isuprel,' 0.3 mcg./kg., was injected into the marginal ear vein. Time interval in minutes.

B. Isolated guinea pig ileum. The segments record the effects of No. 1554, No. 1590 and 'Isuprel,' respectively. Time intervals in minutes.

Both inhibition and excitation are obtained with 'Epinine,' nor-epinephrine, epinephrine and No. 1516, inhibition being more easily elicited than excitation. The homologues of nor-epinephrine in which the N-alkyl group is larger than

ethyl show only inhibitory effects. The branched chain butyl derivatives (Nos. 1505 and 1424) equaled 'Isuprel' in their effects and were much more effective than the *N-n*-butyl derivative. The *N*-isopropyl homologues of tyramine (No. 1554) and 'Kephrene' (No. 1590) were inhibitory in comparatively low dilution. Those compounds that were found most effective in lowering blood pressure and in producing bronchodilatation were found to be most effective in relaxing the isolated guinea pig ileum.

Isolated uteri of rabbits and guinea pigs were suspended in the muscle bath as described above for the guinea pig ileum. The effect of various concentrations of these sympathomimetic agents was determined in the usual manner. The results obtained are shown in table 4 and figure 5. Excitation was obtained with 'Epinine,' nor-epinephrine, epinephrine, and No. 1516. 'Kephrene' inhibited the guinea pig uterus but excited the rabbit uterus. The larger *N*-alkyl homo-

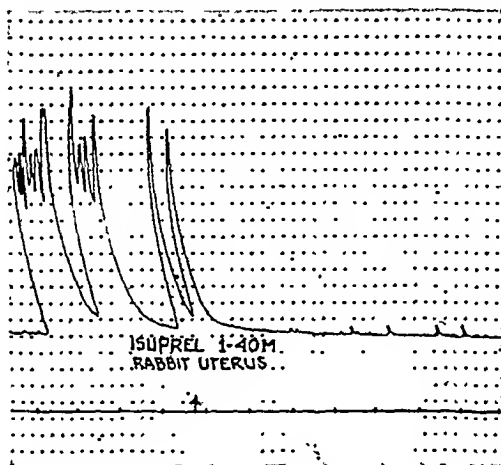


FIG. 5. EFFECT ON THE ISOLATED NON-GRAVID RABBIT UTERUS
'Isuprel' was added to the bath to make a dilution of 1:40,000,000

logues of nor-epinephrine relaxed the uterus and inhibited motility. No. 1960, the *N-n*-butyl homologue, was found to be less effective than the branched chain butyl homologues, Nos. 1505 and 1424. 'Isuprel,' the *N*-iso-propyl homologue of nor-epinephrine, appears to be the most effective uterine spasmolytic agent, with the *N-sec*-butyl and *N-tert*-butyl homologues being somewhat less effective. The *N*-isopropyl homologues of 'Epinine' and 'Kephrene' were found to be ineffective in the dilutions used. The results are quite similar to those obtained with the isolated guinea pig ileum in that the compounds found most effective in causing inhibition of motility and tonus were also most effective in relaxing the uterus. Those substances that increased tonus and motility of the guinea pig ileum in relatively low dilution were predominantly excitatory to the uteri of guinea pigs and rabbits.

Toxicity. Acute toxicity was determined in mice by intraperitoneal injection.

All test animals were obtained from our own colony, were injected and were kept under observation in the colony room where the environmental temperature was maintained at 76°F. Deaths occurring within 72 hours were recorded. Distilled water solutions of the hydrochloride or acetate salts were used in all instances. Results obtained are shown in table 5. Epinephrine was found to be the most toxic substance. Racemic epinephrine is approximately 60 per cent as toxic as epinephrine, suggesting that the toxicity of the d, l-mixture results largely from the l-isomer. The N-ethyl homologue of nor-epinephrine is distinctly less toxic than nor-epinephrine or epinephrine. The N-alkyl homologues of nor-epinephrine in which the alkyl group is larger than ethyl have comparatively low toxicities. The N-n-butyl homologue appears to be more toxic than either the N-sec.-butyl or N-tert.-butyl homologues. The N-isopropyl homologues of both 'Epinine' and 'Kephine' were found to be more toxic than either 'Epinine'

TABLE 5

Acute intraperitoneal toxicity in mice of derivatives of hydroxytyramine

COMPOUND	NUMBER OF ANIMALS	TOXICITY*		
		LD ₁₀	LD ₅₀ ± S.E.	LD ₉₀
		mg/kg	mg/kg	mg/kg.
'Epinine'	115	350	770 ± 71.8	1700
1554	42	410	500 ± 21.9	615
Nor-epinephrine	72	4.6	15.6 ± 3.8	53.5
d,l-Epinephrine	80	2.92	7.8 ± 1.3	21
1516	82	11.1	27.5 ± 4.23	67.8
'Isuprel'	273	377	494 ± 13.9	650
1505	74	310	370 ± 11.5	443
1424	76	410	464 ± 8.6	520
1960	59	278	347 ± 12.2	432
'Kephine'	73	750	902 ± 25.3	1090
1590	56	387	470 ± 15.6	570
Epinephrine ..	80	2.15	4.6 ± 0.55	9.9

* Calculations were made according to the method of Miller and Tainter (7).

or 'Kephine.' This is in marked contrast to the results obtained with nor-epinephrine and its N-isopropyl homologue ('Isuprel').

In order better to describe the toxicity of this series of drugs, the L.D.₁₀, L.D.₅₀ and L.D.₉₀ values were determined by the method of Miller and Tainter (7) and are shown in table 5. Arrangement of the drugs according to their toxicity gives approximately the same order in each column. Results obtained with 'Epinine' suggest that this substance has a lower L.D.₁₀ and a higher L.D.₉₀ than would be expected from the L.D.₅₀ value. Results obtained with this drug were quite variable, as suggested by a mean of 770 ± 71.8 mg./kg. Although 'Isuprel' and No. 1554 have approximately the same L.D.₅₀, 'Isuprel' is distinctly less toxic than No. 1554 at the L.D.₉₀ dose. 1-Epinephrine is most toxic according to each of the three criteria, and 'Epinine' and 'Kephine' are least toxic with respect to the L.D.₅₀ and L.D.₉₀ values.

DISCUSSION. In previous publications (2, 4) from this laboratory, it has been suggested that 'Isuprel' is a sympathin I-mimetic agent in which the inhibitory actions of epinephrine are enhanced. The importance of the structure of the N-alkyl group for this type of action was pointed out originally by Lands, Rickards, Nash and Hooper (8), in connection with a series of tyramine derivatives. We have been able to broaden this investigation by the use of a comparable series of hydroxytyramine derivatives. Similar results have been obtained with both series of compounds. However, in the case of the hydroxytyramine derivatives, the N-*tert.*-butyl homologue was found to equal or to exceed slightly the activity of the N-isopropyl derivative ('Isuprel') as a vasodepressor agent. If the average doses given in table 1 are corrected for the difference in the molecular weights of the compounds, the N-*tert.*-butyl homologue (No. 1505) is about 20 per cent more vasodepressor than 'Isuprel.'

Epinephrine-like vasodepressor responses have been described for 3,4-dihydroxyephedrine by Schaumann (9). This substance is reported to be depressor in small and pressor in large doses. The action of the primary amine, 3,4-dihydroxynorephedrine ('Cobefrine'), is predominantly pressor (Schaumann, 9; Tainter, 10). The next higher homologue, 1-(3,4-dihydroxyphenyl)-2-amino-1-butanol ('Butanefrine') has distinct vasodilator effects (Tainter, 10). Cameron, *et al.* (11) have reported that this effect results from stimulation of both cholinergic and adrenergic vasodilators. It seems not improbable that the vasodilatation obtained with the above compounds and that obtained with the hydroxytyramine derivatives described here result from stimulation of adrenergic vasodilators. Inasmuch as complete atropinization did not significantly alter the magnitude of the fall in blood pressure obtained in our experiments, it would appear that cholinergic vasodilatation did not contribute to this effect.

The addition of the N-methyl group to nor-epinephrine diminishes pressor potency and, under suitable conditions, vasodilatation may be the dominant effect. Neither hydroxytyramine nor its N-methyl homologue, 'Epinine,' caused a fall in blood pressure. Similarly, 'Kephrene' was found to be pressor, no epinephrine-like depressor effects being obtained in our experiments. Barger and Dale (12) have reported 1-(3,4-dihydroxyphenyl)-2-ethylaminoethane to be more pressor than hydroxytyramine. The corresponding analogue, 1-(3,4-dihydroxyphenyl)-2-ethylaminoethanol (No. 1516), is pressor, with strong depressor effects being easily demonstrated. The alcoholic hydroxyl on the side chain appears to be important for depressor effects with compounds wherein pressor effects are distinct.

Both excitatory and inhibitory effects can be demonstrated in other sympathetically innervated organs containing smooth muscle. All substances, except 'Kephrene,' which caused a rise in blood pressure, also induced contraction of the isolated guinea pig ileum, when present in sufficiently high concentration. With rabbit and guinea pig uteri, vasoconstrictor compounds were predominantly excitatory, whereas vasodilator compounds were predominantly inhibitory.

Marrazzi (13) has reported that epinephrine causes depression of the response of the sympathetic ganglion of the cat. This investigator has reported more

recently (14) that epinephrine is more inhibitory than nor-epinephrine or 'Epine.' Marrazzi and Marrazzi (15) found that the inhibition of synaptic transmission did not parallel the pressor effects of sympathomimetic amines

The evidence suggests that these inhibitory (sympathin I) effects have a common physiological basis and that the structural requirements for an effective inhibitory (sympathin I-mimetic) drug are as specific as those described for excitatory (sympathin E-mimetic) effects. Both the presence of an alcoholic hydroxyl on the side-chain and a branched N-alkyl group appear to be important structural requirements for inhibitory actions induced by the hydroxytyramine derivatives described here.

SUMMARY

1. Racemic nor-epinephrine is distinctly more pressor than racemic epinephrine.
2. Inhibitory (sympathin I-mimetic) actions of nor-epinephrine are increased by N-alkyl substitution.
3. The structure of the N-alkyl group is an important factor influencing inhibitory potency.
4. Inhibitory effects are not prominent with those hydroxytyramine derivatives that do not contain the alcoholic hydroxyl of nor-epinephrine.
5. The significance of these findings is discussed.

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RESPIRATORY, ELECTROENCEPHALOGRAPHIC, AND BLOOD GAS CHANGES IN PROGRESSIVE BARBITURATE NARCOSIS IN DOGS¹

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Unconsciousness as the result of an overdose of a barbiturate drug is becoming a familiar problem to the clinician. Usually the diagnosis is rendered easy by a history of ingestion of sleeping tablets. The depth of narcosis, however, is apt to be difficult to determine. The blood pressure, reflex changes, the response to painful stimuli, and size of pupils are helpful, but may be confusing and conflicting. The complications of barbiturate intoxication, namely hypoventilation and pulmonary atelectasis (Swank and Smedal) may be equally misleading. They cause an increased secretion of fluid into the air passages, the result of hypoxia and hypercapnia (Drinker, 1945); and a falling blood pressure and further depression of respirations. Contrarily, attention has been drawn to the fact that hypoxia may also stimulate breathing and mask serious barbiturate depression. (Beecher and Moyer, 1941.)

Since the usual criteria for judging the depth of barbiturate narcosis clinically are frequently unreliable, further investigation of the physiologic changes which occur during narcosis are indicated. In the present study the brain waves, respirations, and other phenomena have been observed during increasing narcosis. With this combined information it has been possible to judge the depth of narcosis with a degree of accuracy not attained by clinical methods alone.

MATERIAL AND METHODS. A total of 55 dogs weighing 8 to 20 kgs. were used. In most experiments, sodium amytal³ was used for narcosis; the initial dose was 0.05 to 0.06 gms. per kilogram intraperitoneally. All subsequent barbiturates and analeptics were given intravenously. In some experiments nembutal was used in place of, and given by the same route as sodium amytal. The doses were approximately half as large as for sodium amytal. The animals were prepared as follows: Tracheal and venous cannulae were tied in place. A pair of stimulating electrodes contained in rubber tubing were placed on one sciatic or saphenous nerve, and held in place by closing the defect in the muscles and skin. The stimulating current consisted of 60 cycle alternating current which was adjusted to just supramaximal for muscular contraction during very light narcosis. The nerve was tied and crushed distal to the electrodes. Respirations were measured from the chest by means of a rubber bellows and recorded continuously by an ink-writing recorder. Abdominal respirations were measured in most experiments by means of a balloon contained in a cloth sack wrapped around the abdomen and recorded in the same manner. Minute volume of in-

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³ The sodium amytal was furnished by Eli Lilly and Co., and nembutal and heparin by Abbott Laboratories.

spiration was measured by 8 liter gasometers with flutter valves in the system near the tracheal cannula to prevent rebreathing. Brain waves were recorded from the intact scalp by small electrodes held in place by collodion, directly from the cortex by silver wires, and from subcortical regions by coaxial electrodes. Bipolar recording was used throughout and the brain potentials were amplified and recorded by push-pull of a Grass ink-writer electroencephalograph. Arterial blood pressure was measured from the femoral artery by means of a mercury manometer. Clotting in the system was prevented by a 0.03% solution of heparin. Right auricular pressures were measured by a catheter in the femoral vein extending to the right auricle of the heart. The baseline for this was arbitrary, and only changes in the pressure were noted. The arterial O_2 and CO_2 contents were determined by the combined method of Van Slyke and Neill. The bloods were obtained under oil from a cannula in the femoral artery and prevented from clotting by potassium oxalate or heparin. All bloods were analyzed immediately after they were drawn.

The narcosis was altered as follows: After each set of measurements, further sodium amytal was injected slowly via the venous cannula which was kept patent by a constant slow infusion of normal salt solution. The amount of each of these repeated injections varied, but the following were found most suitable for gradually increasing the narcosis: for a 10 kg. dog the dose of sodium amytal was 0.05 gms.; for a 13 kg. dog 0.1 gm.; for a 16 to 17 kg. dog, 0.15 gms.; and for a 20 kg. dog, 0.2 gms. If the injection entered the circulation slowly there was a uniformly gradual decrease in the depth and/or frequency of breathing, and also in the amplitude and frequency of the brain waves. When injections were made near the junction of moderate and deep narcosis, the appearance of "blackouts" in the electroencephalogram and absence of increased ventilation on inhalation of 10 per cent CO_2 (these changes and relationships are described later in this paper) functioned as very sensitive "indicators". By repeating these observations frequently it was possible to ascertain not only the time it took for maximum narcosis to develop, but also to determine the duration of effect, and particularly the time at which recovery from the injection of the drug started. A maximum depression of respirations and brain waves was produced in 4 to 5 minutes. This state remained fairly uniform for the next 15 minutes. In the ensuing 5 minutes significant functional return could be detected. By injecting sodium amytal in proper doses every 20 minutes and waiting 5 minutes after each injection, 15 minutes of fairly uniform narcosis was available for study. To guard against the inevitable small differences in depth of narcosis in the test period, 10 per cent CO_2 , 10 per cent O_2 , 100 per cent O_2 , and sensory nerve stimulation were tested in varying sequence.

After preparation of the animals, basic measurements were made. These included minute volume of inspired air, a sample of the brain waves, arterial blood pressure, right auricular pressure and a sample of arterial blood for its O_2 and CO_2 contents. The responses of the animal's respirations to 10 per cent CO_2 (with 20 per cent O_2), to 10 per cent O_2 (in nitrogen), to 100 per cent O_2 , to electrical stimulation of the sciatic or saphenous nerve were determined in nearly every experiment. These determinations were repeated as the narcosis deepened. In selected experiments the analeptic drugs were tested. With few exceptions only one was tested in each experiment.

RESULTS. The results will be described in four sections. The first section will describe the changing character and responsiveness of the respirations and brain waves to the inhalation of 10% CO_2 , and 100% O_2 , and to stimulation of sensory nerves. Section II will consider the general clinical and reflex changes and arterial and venous pressures as the depth of narcosis is increased. Section III will consider the O_2 and CO_2 contents of the blood at varying depths of narcosis. Section IV will consider the effects of caffeine, aminophyllin, metrazol, picrotoxin, and benzedrine on the respirations and brain waves at different levels of narcosis.

I. The character of the respirations and brain waves, and their responsiveness to 10% CO₂, 10% O₂, and to sensory nerve stimulation at different depths of narcosis: Our physiological data has made it convenient to divide progressive narcosis into three stages—light, moderate, and deep narcosis. When preparations for experimentation were completed the dogs were in a state of light narcosis, (see figure 5, A to B). They responded to painful stimuli with a vigorous increase in respirations, and by withdrawing weakly. The corneal and tendon reflexes were brisk. The breathing of 10% CO₂ and electrical stimulation of the sciatic and saphenous nerves caused a brisk and sustained increase in respirations. The inhalation of 100% O₂ was followed by slight if any depression of respirations. The brain waves were well organized and usually slower than normal. In most instances, bursts of 10/second sharp waves were observed. During this period slight changes in narcosis resulted in marked alterations in respirations and brain waves.

Moderate narcosis is represented in figure 5, B to C. Respiratory stimulants produced a less vigorous response, and 10% CO₂ became a less potent stimulant than 10% O₂. One hundred per cent O₂ caused a definite although slight depression of respirations, and the brain waves gradually lessened in amplitude and frequency.

Deep narcosis (figure 5, C to the end) was attended by two phenomena with a nearly simultaneous onset; first, respirations no longer increased when 10% CO₂ was inhaled; and, second, the brain potentials showed periodic complete suppression ("blackouts") separated by periods of slow waves, some of high voltage. The respirations continued to be increased slightly by sensory nerve stimulation and by 10% O₂, and the depression of respirations by 100% O₂ was greater than during moderate narcosis. A further slight increase in narcosis was attended by depression instead of stimulation of breathing by 10% O₂, by a complete absence of increased ventilation after sensory nerve stimulation, and by rapidly deepening cyanosis, falling blood pressure, and death.

A more detailed account of the various changes just enumerated will now follow.

a) *Character of the respirations:* During light narcosis breathing was accomplished by a combination of costal and diaphragmatic action. The costal movements were vigorous during light narcosis, but gradually weakened during moderate narcosis and were absent during part, and occasionally during all, of deep narcosis (figure 1). Breathing during deep narcosis appeared to be almost entirely diaphragmatic. A uniformly gradual slowing of the respiratory rate also occurred during light, moderate and the first part of deep narcosis. This was followed by a rapid decline in the respiratory rate just before death.

In eight cases the minute volume of inspired air was measured. In very lightly narcotized dogs (figure 5, A) this was observed to vary between 300 and 500 cc./kg. The pulmonary ventilation decreased rapidly with increasing narcosis during the light stage to reach 160 ± 30 cc./kg. (figure 5, B). This marked the onset of moderate narcosis. During moderate narcosis the minute volume fell more slowly and reached 110 cc. ± 12 cc./kg. at the onset of deep narcosis.

At this point 10% CO_2 failed to increase respirations (see figure 1, E), and periodic "blackouts" appeared in the brain waves. Further narcosis resulted in a continued decline in the minute volume due principally to further slowing in the rate of respirations (see figure 1).

b) *Stimulation of respirations by 10% CO_2 , 10% O_2 and sensory nerve stimulation:* In light narcosis 10% CO_2 increased the ventilation by from 100% to 200%, 10% O_2 about half this much, and sciatic or saphenous nerve stimulation by 300% or more (figure 5, A to B). Sciatic nerve stimulation and 10% CO_2 caused an increase in both the depth and rate of respirations; and 10% O_2 an increase

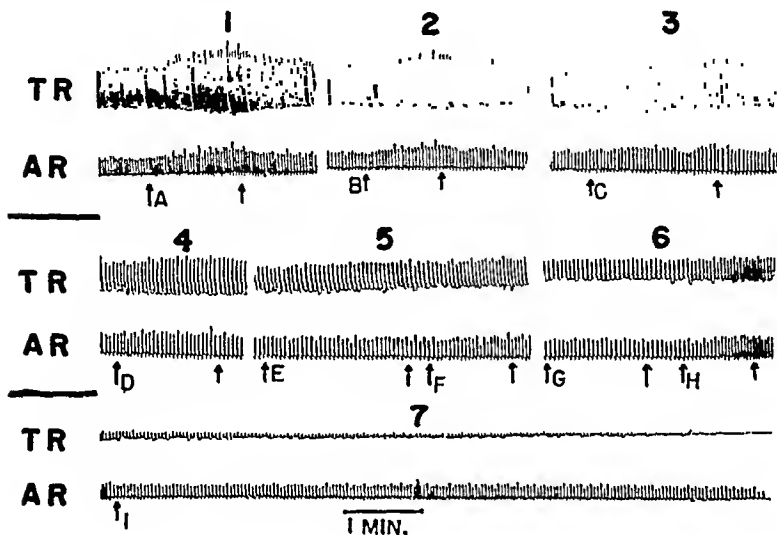


FIG. 1

Thoracic (TR) and abdominal (AR) respirations in a dog weighing 10.6 kg. The numbers above the individual records refer to successive steps in the increasing narcosis. Between each record, except between 6 and 7, sodium amytal 0.1 gm. was given by slow I.V. infusion. At A, B, C, D, E, and G the dog was given 10% CO_2 and 20% O_2 in nitrogen to breathe. Note the progressive diminution in the response of the respirations to CO_2 , until at E and G there is no evident response. Also note the reduction of thoracic and the relative preservation of abdominal respiration in deep narcosis. This was a constant finding.

primarily in the rate. The increased ventilation lasted through the period of stimulation, which was usually 1 or 2 minutes for hypercapnia and hypoxia, and 30 or more seconds for electrical stimulation of the sciatic or saphenous nerve.

With the deepening of narcosis, but still in the light stage, the increased ventilation resulting from the inhalation of 10% CO_2 decreased rapidly. During moderate narcosis this decrease continued, only more slowly. The response to 10% O_2 , although not so great early in light narcosis, lessened more slowly than the response to CO_2 . As a result, during moderate narcosis respirations were increased more by the inhalation of 10% O_2 than by 10% CO_2 , and by the end of

this period 10% CO₂ no longer increased breathing. 10% O₂ continued to stimulate breathing until late in deep narcosis (figure 1, F and H). The response to sensory nerve stimulation dropped off sharply during light and moderate narcosis and in deep narcosis paralleled the response to hypoxia (figure 5, "C" to end).

The nature of the respiratory increases after various stimulations also changed. Whereas the response to 10% CO₂ was prompt and sustained early in light narcosis, late during moderate narcosis the onset was delayed (figure 1) and the initial increase in minute volume was followed by a decline to the near pre-stimulation level. In late deep narcosis the increase in respirations caused by 10% O₂ and sensory nerve stimulation also failed to be sustained, and there was in addition a greater delay in the onset of increased ventilation following these stimulations. Finally, in very deep narcosis 10% O₂ caused a decrease rather than increase in the speed and depth of breathing, and then death followed, ushered in by failure of respiration. Sometimes this decrease in ventilation was preceded by a short initial increase in the rate and depth of respirations which was then followed by slowing and cessation of breathing.

Beecher and Moyer (1941) state that the stimulation of respirations by hypoxia (in this case, 5% O₂) results in the same minute volume respiration throughout a wide range of narcosis, even though the minute volume respirations prior to the rebreathing may differ. Our observations indicate that hypoxia stimulated respirations over a wide range of narcosis, but that as the narcosis deepened the attained minute volume of respiration became less (see figure 5).

c) *Depression of breathing by 100% O₂*: The depressing effect of 100% O₂ on the respirations of barbiturate narcotized animals is well known from the original observations of Mosso (1904) and the detailed studies of Marshall and Rosenfeld (1936). In very light barbiturate narcosis we noted very little, if any, depression as did Dumke, Schmidt, and Chiodi, 1941. As the narcosis deepened, 100% O₂ caused a progressively greater depression of respirations, and this often reached alarming proportions in our deep narcosis. We did not observe the cessation of breathing in deep narcosis which is claimed by Marshall and Rosenfeld (1936) and Beecher and Moyer (1941) to occur when 100% O₂ is breathed. Our observations indicate that this happens only in very deep narcosis. Breathing is then in a precarious state and may stop spontaneously. We did observe apnea of 4 to 5 minutes' duration after artificial respirations with O₂ in deep narcosis. In the experiments in which this was observed breathing was re-established spontaneously. A diffusion of oxygen to the lungs from the trachea, tubing and gasometer was not an important factor in prolonging the period of apnea after artificial respiration with oxygen, since when the artificial respirations were stopped the connection with the source of oxygen was interrupted at the tracheal cannula. Furthermore, as pointed out by Draper and Whitehead (1944), the accumulation of carbon dioxide in the alveoli when the mechanical action of breathing is absent constitutes another obstruction to the absorption of oxygen by the lungs.

d) *The Electroencephalogram*: We did not record the brain waves prior to the administration of sodium amytal. In a few instances the narcosis was quite

light when the E.E.G. record began and in one of these the electroencephalogram was dominated by waves of about 25/second frequency. In others, very early in light narcosis periodic bursts of about 10/second sharp waves were observed. Figure 2, A to D, shows a characteristic series of electroencephalograms recorded from the dura overlying the motor cortex. Note the bursts of about 10/second sharp waves, one of which is underlined by a dashed line in light narcosis (tracing

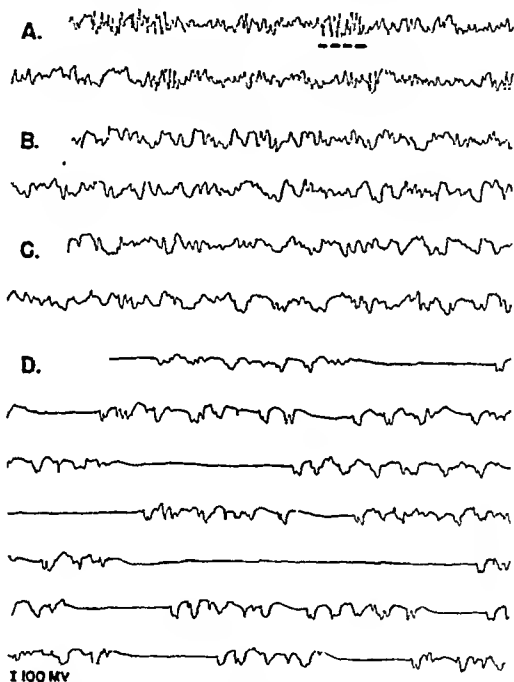


FIG. 2

EEG records from the dura over the frontal pole in 4 stages of increasing narcosis. Each line represents 10 seconds of recording. Record A taken about 45 minutes after the dog has been given 0.06 gms. sodium amytal per kg. intraperitoneally. About 5 minutes prior to the samples taken at B, C, and D, the narcosis was increased by giving 0.1 gm. sodium amytal intravenously. In A, note the bursts of 10/second high voltage sharp waves, one of which is underlined. In B and C, the frequency of the brain waves is decreased and at D, there are short intervals in which no electrical activity could be recorded ("blackouts"). D is a continuous strip of record 70 seconds long.

A). Note also the gradual slowing of the brain waves as the narcosis deepened during moderate narcosis (B and C), and the appearance of periods of inactivity in the electroencephalogram during deep narcosis (D). These periods of suppression were 1 to 5 seconds long at first. They became longer as the narcosis deepened, and finally just before death, continuous suppression was present. The periods of suppression ("blackouts") were also recorded on a cathode ray oscillograph in order to make sure that very fast activity was not present in the

quiet intervals. With high amplification no electrical activity was recorded directly from the cortex of different areas during "blackouts" when the two electrodes were separated by a distance of 5 mm. or less. In all instances the cortex became electrically inactive continuously before the breathing and the

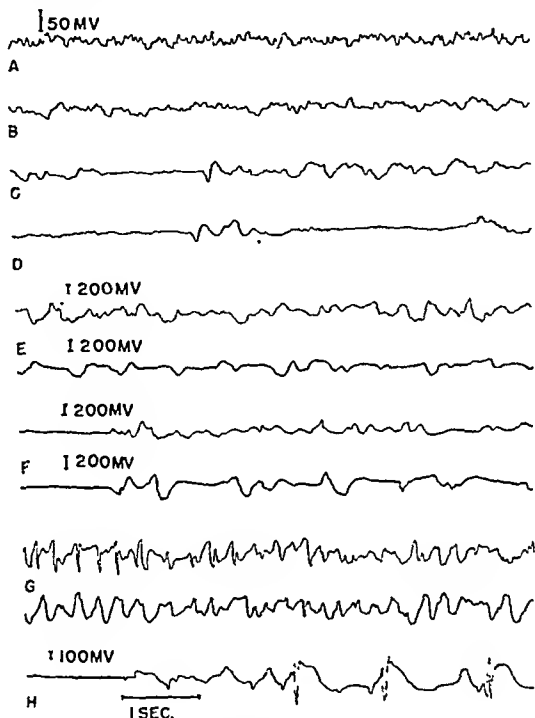


FIG. 3

A, B, C, D,—Sample electroencephalogram from scalp leads in 4 successive stages of increasing narcosis in the same dog. E, F, and G, (upper and lower tracings),—direct recording from the cortex. The upper tracing was from the motor cortex (for the hind leg) and the lower tracing from a point 2.5 cm. posterior to this point. Note that while continuous activity persists in the motor strip in E the posterior lead is beginning to show flattening of the base line. In F the "blackouts" are established. Record G was taken 18 minutes after 9 mgs. of picrotoxin had been given intravenously. Note the spike activity from the motor strip and the slower potentials from the posterior cortex. H. Another animal. Direct bipolar record from cortex with a pledget soaked in picrotoxin placed between the recording electrodes. Note that the spikes do not occur during the "blackouts".

heart stopped. Figure 3, A to D, illustrates the character of the brain waves, from light to deep narcosis, when recorded from the intact scalp.

When recording directly from the cortex short periods of suppression appeared first in the posterior cortex during moderate narcosis. In these periods, the brain waves continued without interruption in the motor cortex and the cortex anterior to it. Later, at the beginning of deep narcosis, periods of suppression

("blackouts") appeared in the motor cortex. The duration of these increased with narcosis, and they were accompanied by synchronous periods of suppression in all cortical areas of both hemispheres. Each "blackout" from the motor cortex terminated periodically in a burst of high voltage slow activity with intermingled sharp waves and spikes (figure 3, E and F), which was accompanied by almost simultaneous electrical activity in all cortical areas.

The close correlation of the "blackouts" with a failure of the pulmonary ventilation to increase when 10% CO₂ was breathed is worthy of further comment. In one instance, impaired circulation to one hemisphere of the brain, apparently the result of damage to the veins draining into the superior longitudinal sinus, was attended by the appearance of the "blackouts" in this hemisphere early in moderate narcosis. The normal relationship between the "blackouts" and a failure of ventilation to increase when 10% CO₂ was breathed persisted, however, in the undamaged opposite hemisphere.

In six experiments, dogs exhibiting "blackouts" of their brain waves were artificially respired with 100% O₂. This procedure returned the O₂ content of the blood to or near normal, where it remained during artificial respiration. The arterial blood pressure usually fell slowly and steadily during artificial respirations, often to a mean pressure of 30 to 40 mm. In spite of the falling blood pressure, the "blackouts" were abolished by artificial respirations with 100% O₂ and continuous brain wave activity was restored. This was surprising in view of the observation by Beecher, McDonough, and Forbes (1938) that lowering the blood pressure to approximately this same level in ether or pentobarbital anesthetized animals caused flattening of the brain waves, and at still lower mean blood pressures the brain waves disappeared until the blood pressure was elevated again. In experiments in which the "blackouts" had been abolished by artificial respiration with O₂, additional sodium amytal caused the "blackouts" to re-appear. Also the "blackouts" reappeared when the artificial respirations were stopped and cyanosis was allowed to develop again. When the "blackouts" were caused to reappear in artificially respired animals by additional sodium amytal, voluntary breathing often did not occur when the artificial respirations were discontinued. This was probably due to the very large amount of sodium amytal which these animals had received. In other animals voluntary respirations did appear, after delays of as long as 4 to 5 minutes, but at a very slow rate. It was clear that artificial respiration was no more than a temporary expedient. It appeared from these observations that an adequate pulmonary ventilation with oxygen delayed the appearance of the "blackouts" in progressively deepening narcosis, and conversely, that hypoxia contributed (with sodium amytal) to the production of the "blackouts".

II. Additional General and Reflex Changes, and Arterial and Venous Pressures: The mean arterial blood pressure was determined in only 6 experiments, and was not recorded continuously. It varied from 90 to 130 mms. of Hg during light narcosis. As a rule this pressure fell slowly and steadily during light, moderate, and the early part of deep narcosis to reach a mean of 70-80 mms. of Hg. Later in deep narcosis, near the terminal drop in pulmonary ventilation and just before

death, the arterial blood pressure fell rapidly. The breathing of 10% O₂ caused a temporary increase in arterial pressure of from about 10 to 30 mms. of Hg., and 10% CO₂ caused a less moderate drop in blood pressures. In two experiments electrical stimulation of the sciatic nerve caused a sharp rise in arterial pressure in light narcosis and insignificant changes in late moderate and deep narcosis. The venous pressure remained unchanged or varied inconsistently.

The corneal reflexes weakened rapidly and disappeared at about the mid-point of moderate narcosis. The patellar reflexes were still active at this point, but were no longer obtained near the beginning of deep narcosis. These changes and the blood pressure changes were not consistent, however.

During light narcosis the cortex, when exposed, had a good color, and no tendency to herniation was noted. Pulse artifacts were usually absent in the encephalograms. During moderate narcosis very slight cyanosis developed and the brain appeared full. Herniation of the brain was absent and pulse artifacts were absent or negligible in the brain waves. During deep narcosis marked changes were observed in the exposed brain. It became deeply cyanosed and greatly swollen, and rapidly herniated through the opening in the dura. First there was marked pulsation of the brain, but when the herniation increased this disappeared. Restoration of normal pulmonary ventilation by picrotoxin or by artificial respiration caused a rapid disappearance of the cyanosis, shrinkage of the brain sufficient to reduce its herniation, and return of the arterial and respiratory pulsations. The "blackouts" in the EEG were not related to herniation of the brain just described since they were recorded in experiments in which the skull and dura were intact.

III. The O₂ and CO₂ contents of arterial blood during progressive narcosis: The changes in O₂ and CO₂ contents of the blood reflected the degree of depression of the respiratory mechanisms. The figures for O₂ and CO₂ contents were obtained from 8 experiments. In four of these experiments many points on the curve were determined, together with respiratory measurements, as shown in figure 4. In the remaining experiments only 2 or 3 points on the curves together with respiratory data and brain waves were determined. The combined data is shown in figure 5. In this figure the individual variations when breathing air are indicated as outlined by the vertical lines. The variations for 10% CO₂ and O₂ were similar and for electrical stimulation of the sciatic nerve often greater (see Section I, a and b, this paper). However, in individual experiments the relative minute volume respirations when breathing air, 10% O₂, 10% CO₂, and during stimulation of the sciatic nerve were as indicated. It should be noted first that the O₂ and CO₂ levels bore a reciprocal relationship to one another; as the O₂ level fell, the CO₂ level rose. Second, there were two sharp changes in the O₂ and CO₂ levels in the arterial blood during progressive narcosis. In light narcosis the O₂ level fell abruptly from about 90% (at "A") to roughly 70% saturation (at B). This was accompanied by a corresponding rise in CO₂ content from about 35.0 vols./100 cc. to 43.0 vols./100 cc. During moderate narcosis (from B to C) there was a continued slow drop in O₂ to about 55% saturation and an equally slow rise in CO₂ to about 45 vol./100 cc. at which time ("C")

"blackouts" became established. During deep narcosis there occurred a second sharp fall in O_2 content and a corresponding rise in CO_2 content. All of the oxygen determinations are included in figure 5; each one is indicated by a circle. As can be seen, fairly uniform results were obtained. The same number of points were determined for CO_2 , but these varied more than the oxygen determinations. The CO_2 determinations are not included in the figures.

The initial sharp fall in O_2 and rise in CO_2 content during light narcosis ac-

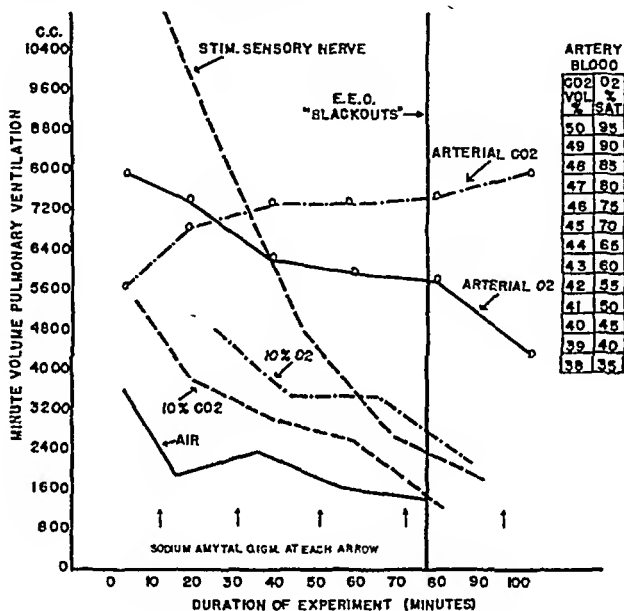


FIG. 4

Diagrammatic representation of an individual experiment showing the various factors involved. The experiment was considered to have begun about one hour after the dog had been given 0.06 gms./kg. of sodium amytal intraperitoneally. The arterial CO_2 and O_2 were plotted against the columns in the right side of the diagram. The points for air, 10% CO_2 , 10% O_2 , and sensory nerve stimulation refer to the minute volume of inspired air or gas. Note the reciprocal relationship of the O_2 and CO_2 , and the increasing approximation of responses to 10% CO_2 , 10% O_2 , and sensory nerve stimulation to the figure for air as the narcosis deepened. Note also the correlation between the establishment of the EEG "blackouts" and the failure of 10% CO_2 to increase the ventilation.

accompanied a sharp decrease in the responsiveness of respirations to 10% CO_2 . As a result, the response of respirations to 10% CO_2 was less than to 10% O_2 during moderate narcosis. The grossly irregular respirations which often developed late in light narcosis also followed this initial abrupt change in O_2 and CO_2 content in the arterial blood. The second abrupt fall in O_2 and rise in CO_2 content in the arterial blood which occurred during deep narcosis was attended by an absence of increased ventilation during the inhalation of 10% CO_2 , by a decreasing response of respirations to 10% O_2 and sciatic nerve stimulation, and by the presence of "blackouts" in the brain waves.

IV. The effect of caffeine, aminophyllin, metrazol, picrotoxin, and benzcdrine on the respirations and brain waves during progressively deepening narcosis:

a) The general effect of increasing narcosis was to decrease progressively the effectiveness of the pharmacological respiratory stimulants listed above. Caffeine (0.25 to 0.5 gms.; 7 observations in 3 experiments) produced slight and transient stimulation in light narcosis, and no change in moderate or deep narcosis. Aminophyllin (0.6 to .12 gms.; 8 observations in 5 experiments) was definitely more effective than caffeine. It stimulated respirations throughout both light and moderate narcosis, but was without effect in deep narcosis. Also the effect

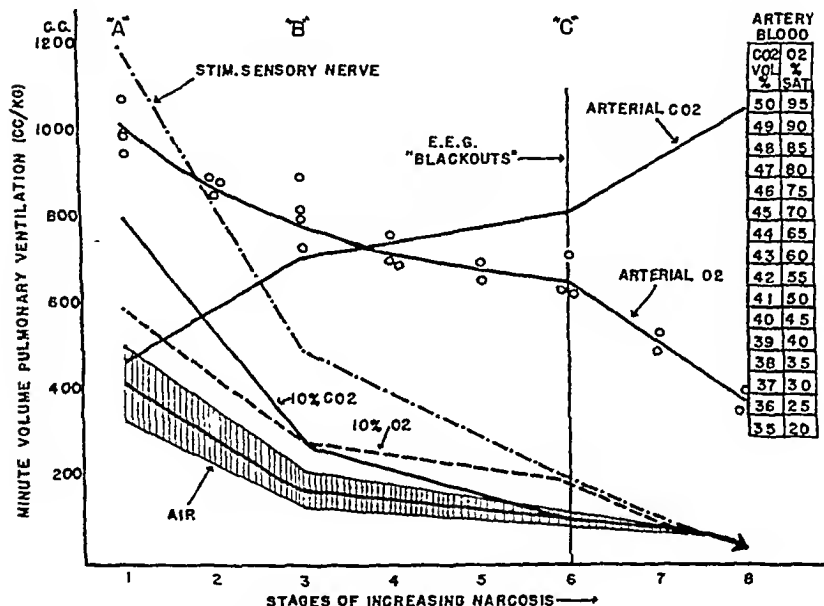


FIG. 5

A composite diagram of eight experiments of the type represented in figure 4. The minute volume of pulmonary ventilation is expressed in cc./kg. of dog weight to make correlation possible. From "A" to "B" is light narcosis, from "B" to "C" moderate narcosis, and from "C" to the end is deep narcosis. Only the points for arterial O₂ determinations were recorded; CO₂ determinations were made from the same samples.

of aminophyllin was sustained; often a single dose stimulated respirations for as long as 30 minutes. Neither of these agents caused a detectable change in the brain waves.

b) Metrazol (0.1 to 0.2 gms.; 18 observations in 7 experiments) stimulated respirations much more vigorously than caffeine or aminophyllin. In general the response to metrazol was immediate and of short duration (15 minutes or less). Metrazol also rendered the respiratory mechanisms more sensitive to physiologic stimulation by 10% CO₂ and 10% O₂, and less responsive to the depressant effects of 100% O₂. However, as the narcosis deepened, the response

to metrazol lessened and in the deeper phases of deep narcosis it failed to stimulate respirations, in the dose employed (figure 1; I).

In our narcotized dogs, metrazol never caused convulsions in the dosage given. It caused moderate increases in the frequency and amplitude of the brain waves which usually preceded the increased respirations, but convulsions, and "convulsive electrical activity" in the encephalogram was never observed. This was probably due to the dosages which were used.

c) *Picrotoxin* (3 to 12 mgs.: 15 observations in 7 experiments) stimulated respiration vigorously during light, moderate, and early part of deep narcosis. Tonic extensor spasms or convulsions accompanied brisk increases in ventilation, but these were usually avoided. In subconvulsive doses picrotoxin rendered the respiratory mechanisms increasingly sensitive to 10% CO₂ and 10% O₂ and reduced the depressing action of 100% O₂. As a result the pulmonary ventilation was increased. Increasing narcosis decreased the stimulating effect of picrotoxin, but as long as 10% O₂ and sensory nerve stimulation increased respirations, picrotoxin did also. The action of picrotoxin could be delayed as long as 30 minutes, particularly in deep narcosis, but the action when present was prolonged. In the doses which we employed picrotoxin failed also to stimulate respirations in advanced deep narcosis, and on at least two occasions was attended by a cessation of respirations.

Picrotoxin changed the brain waves remarkably. It increased the frequency and amplitude of the cortical potentials and abolished the "blackouts" early in deep narcosis (figure 3, G). Prior to and during the convulsions, spikes appeared in the electroencephalogram. These spikes appeared first in the motor cortex and in some instances were seen only in the motor cortex. In others they spread to the remaining cortex. In advanced deep narcosis the change in brain waves was slight or absent. It is of interest that significant speeding up of the brain waves always preceded an increase in respirations, and occasionally the brain wave changes were not accompanied by any subsequent increase in breathing. In one instance picrotoxin was applied to the motor cortex between the two recording silver wires by means of a small piece of filter paper. Eighteen minutes later the record shown in figure 4, H was taken. The "blackouts" were preserved, but spike activity appeared during the interval bursts of brain waves.

DISCUSSION. In the present study the electroencephalographic changes and their relationship to respirations appear to be the more interesting observations. Other investigators (Derbyshire, Rempel, Forbes, and Lambert, 1936; Beecher and McDonough, 1939; Hoagland, Himwich, Campbell, Fazekas, and Hadidian, 1939; and Heinbecker and Bartley, 1940) have described the slowing and general flattening of the encephalogram as the narcosis deepens, but the periodic complete suppression ("blackouts") of electrical activity of the cerebral hemispheres for one to many seconds followed by bursts of slow waves with occasional intermingled spikes appears to have been overlooked. This is surprising since they have never failed to appear during deep narcosis in our experiments. Bremer (1937) discussed the relative flattening of the electroencephalogram in barbiturate narcosis, but it is not clear that he referred to the changes described in

this paper. Derbyshire, Rempel, Forbes, and Lambert (1936) also describe the periods of relative quiet in the encephalogram in nembutal narcosis, but it is not clear whether they referred to complete suppression of brain waves or to the state of relative quiet between "nembutal bursts". These bursts are present in light and moderate narcosis in dogs, being absent in deep narcosis when the "blackouts" appeared.

It is well known that the sensitivity of the respiratory center to CO_2 is impaired early in barbiturate narcosis. The development of grossly irregular respirations at the end of light sodium amytal narcosis in some of our animals was probably the initial clinical evidence of this. This was preceded or accompanied by a rapid increase in arterial CO_2 and a rapidly diminishing sensitivity of respirations to 10% CO_2 . As a result, hypoxia stimulated respirations more effectively than hypercapnia, and in all likelihood respirations became controlled primarily by hypoxia. CO_2 probably continued to exert some effect, however, since the pulmonary ventilation and arterial blood O_2 remained fairly stable throughout moderate narcosis. During deep narcosis the inhalation of CO_2 no longer increased breathing and, although hypoxia increased ventilation, it could no longer maintain a sufficiently high ventilation to prevent a serious fall of the arterial O_2 and rise of the arterial CO_2 contents.

We observed that specific changes in the electroencephalogram from the cerebral hemispheres accompanied equally specific changes in the CO_2 sensitivity of the brain stem. Bursts of sharp waves (similar or identical to the "nembutal bursts") were observed in the encephalogram early in light sodium amytal narcosis at a time when the sensitivity of the brain stem to CO_2 was rapidly diminishing. This was less dramatic, however, than the relationship in deep narcosis of "blackouts" in the encephalogram with an absence of sensitivity of the respiratory center to CO_2 . These criteria were more dependable as indices of the depth of narcosis than any other employed in this study.

In general all substances which stimulated breathing became less effective, and all agents which depressed respirations became more effective as the narcosis deepened, an observation made by many investigators. When given early enough to produce an increase in respirations the analeptic drugs appeared, as pointed out by Maloney, Fitch, and Tatum (1931), to increase the animals' tolerance for sodium amytal (as measured in our experiments by the total dosage of drug prior to death). However, when dangerously deep narcosis had become established, and respiratory stimulation was most desired, metrazol and picrotoxin were ineffective in the dosages which we employed.

This observation appears to differ in part from the conclusion of other investigators particularly those of Maloney, Fitch, and Tatum (1931), and Werner and Tatum (1939). In general these investigators employed larger total doses of both metrazol and picrotoxin than were employed in the present study, which may explain our failure to increase the respirations in very deeply narcotized dogs. It should be noted, however, that there was one other significant difference between the two groups of experiments. In those of Maloney, Fitch and Tatum (1931), and Werner and Tatum (1939) a careful evaluation of the depth of nar-

cosis was not made, and the analeptic was given before the narcosis had become maximal. From the statement that the analeptic was given intravenously when "gross reflexes were abolished" (Maloney, Fitch and Tatum, 1931) it can be assumed that the analeptic was given in what we have considered light or moderate narcosis. At that depth of narcosis our animals also responded vigorously and quickly, and an increase in their tolerance for sodium amytal was evident.

The experiments of Marshal, Walzl and LeMessurier (1937) are of particular interest because they more nearly simulated the problem as it presents itself clinically. These investigators tested the effect of picrotoxin on oxygen-produced apnea in phenobarbital and other barbiturate narcosis. In their first experiments picrotoxin was given shortly after cessation of breathing in the cases illustrated in their figures 1, 2 and 3. In each instance, respirations were resumed in approximately 3 minutes or less. In our experiments, picrotoxin in the same or slightly greater doses increased respirations as quickly as this only in light, moderate, or early deep narcosis. In very deep narcosis the action of the drug was delayed much longer, sometimes as long as 30 minutes. Furthermore, early in deep barbiturate narcosis in dogs the present authors have seen respirations resumed spontaneously after oxygen-produced apnea of 5 minutes' duration. It would seem possible to the present writers that the re-establishment of breathing by Marshal *et al.* was not due to the picrotoxin, but was spontaneous. In a second group of experiments the loss of the corneal reflex was used by Marshal *et al.* as the index of depth of narcosis. At this point picrotoxin was administered and vigorous respiratory response resulted. This reflex is lost in our moderate narcosis. Furthermore, the minute volume respirations of their dogs (approximately 140 to 300 cc./kg.) would suggest that the narcosis was of moderate depth or even lighter. At this depth of narcosis we also observed that picrotoxin invariably increased the respirations vigorously; also spontaneous recovery was the rule.

As a result of our own observations and a review of the literature we feel that it has yet to be demonstrated that picrotoxin or metrazol stimulate breathing significantly in dogs after very deep narcosis has been established. There can be no doubt, however, that it is effective in the lighter stages of narcosis discussed above. When picrotoxin or metrazol is given one must be prepared for the convulsions which are very apt to occur. These can be controlled by more barbiturate. Furthermore, one must be prepared for the depression of neuronal activity as indicated by placement and similar tests on rats (Dille and Hazelton, 1939), which follows these convulsions; and by death even after the animals have awakened, unless treatment is prolonged for 12 or more hours (Barlow, 1938).

Picrotoxin appears to act by increasing the responsiveness of nerve cells to physiologic and other stimuli. This is shown by two items of evidence presented here. First, this drug made the respiratory center more responsive to ordinary stimulants (10% CO₂ and 10% O₂); and second, when applied directly to the cortex during the EEG "blackouts", no alteration in the periods of suppression were detected, but high amplitude spikes and sharp waves occurred during the intervening bursts of brain wave activity (figure 3, H). In relation to this find-

ing the respiratory center of narcotized cats has been shown to be increasingly sensitive to electrical stimulation after the injection of picrotoxin (Wells *et al.*, 1944). The mechanism by which picrotoxin increases the tolerance for sodium amytal when given early may be as follows. By increasing the pulmonary ventilation it maintains a normal O_2 saturation of the body tissues. As we have seen artificial respiration with 100% O_2 appears to lessen the depression produced by sodium amytal. The mechanism of this antagonism is not clear, but may result from an increased O_2 utilization by the neurons, the result of the increased O_2 tension in the surrounding tissues.

The observations reported in this paper have shown that reflex and other activity of the nervous system is depressed at all levels of the neuraxis simultaneously and progressively by sodium amytal narcosis. There was nothing to suggest that the cerebral cortex is depressed first, and that successively lower centers are affected later as schematized by Etsten and Himwich (1946). Slowing of the frequency of the brain waves was associated with a lessened sensitivity of the respiratory center to carbon dioxide very early in narcosis. The effect of hypoxia and electrical stimulation of a sensory nerve differed greatly in light and moderate narcosis, but was equal in deep narcosis. The disappearance of the knee jerk in the dogs was accompanied by a failure of 10% CO_2 to increase breathing and by the appearance of "blackouts" in the electroencephalogram. These observations suggesting weakened reflex activity in many parts of the nervous system are not surprising in view of the observation by neurophysiologists that nembutal readily blocks the transmission of multisynaptic spinal reflexes in the spinal cords of animals (Eccles, 1946). It has also been demonstrated that the action of picrotoxin and metrazol are general and not limited to the brain stem. This was shown by the increase in cerebral brain wave activity which usually preceded a noticeable increase in respirations and tonic seizures.

CONCLUSIONS

During progressive sodium amytal narcosis in dogs observations were made on the electroencephalogram, respirations, blood gases, and reflex activity. Consistent correlations were observed, which indicated the relative depth of narcosis with a considerable degree of accuracy. The simultaneous occurrence of changes in the encephalogram and respiratory response to 10% CO_2 was particularly striking.

Evidence is presented which suggests that the maintenance of a normal oxygen tension in the arterial blood lessens the depression of neuronal activity by sodium amytal.

Analeptics, including picrotoxin and metrazol were tested at different depths of narcosis.

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THE EFFICACY OF BAL (2,3-DIMERCAPTOPROPANOL) IN THE TREATMENT OF EXPERIMENTAL LEAD POISONING IN RABBITS

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2,3-Dimercaptopropanol ("BAL") was one of a large series of dithiols prepared and studied by Peters, Stocken, Thompson and their associates (1-6) as possible protective and therapeutic agents against the arsenical war gases. These workers demonstrated its marked affinity for arsenicals, and its ability to prevent their toxic effects by removing arsenic from its combination with tissues to form a stable, relatively non-reactive and rapidly excreted BAL-thioarsenite. BAL can be dissolved in peanut oil and benzyl benzoate to form a stable solution suitable for intramuscular injection (7). Earlier reports from this laboratory have described the effects of such a solution on the excretion of arsenic (7-11), and its efficacy in the systemic treatment of arsenic poisoning in both animals (7) and man (8, 9). More recently, it has been shown (12, 13) that in experimental antimony poisoning, BAL exerts a protective action, saving at least 50 per cent of the animals receiving an otherwise lethal dose of fuadin, tartar emetic, anthiomaline, and p-methyl phenylstibonic acid (12). In both arsenical and antimony poisoning, this protective action of BAL was shown to be associated with a marked increase in the urinary excretion of arsenic and antimony (7, 9-12).

The present study was undertaken to determine the therapeutic activity of BAL in experimental lead poisoning. In a similar study, in which rabbits received intraperitoneal injections of solutions of lead nitrate followed by daily intramuscular injections of BAL, Braun, Lusky and Calvery (13) concluded that BAL is not an effective agent for the treatment of either acute or chronic lead poisoning. Animals so treated died in a shorter period of time than those receiving lead alone, suggesting that lead and BAL exerted additive toxic effects. The effect of BAL on the urinary excretion of lead was not determined. More recently, however, Ryder, Cholak and Kehoe (14) demonstrated a significant increase, averaging 34-fold, in the urinary excretion of lead following the administration of BAL to patients with lead colic. Concomitant with the increased excretion, there was a decrease in the concentration of lead in the whole blood, presumably due to the dissociation of lead from its combination with the red blood cells, and probably accounting for a large proportion of the observed increase in the urinary excretion of lead.

A. THE EFFECT OF BAL ON SUBACUTE LEAD POISONING PRODUCED IN RABBITS BY A SUBCUTANEOUS DEPOT. *The effect of BAL on survival.* Animals were given

5 consecutive daily subcutaneous injections of an aqueous solution of 20 per cent lead acetate, at dosages varying from 120 to 240 mg./kg. per injection. Due to the direct toxic action of the lead salt, large indurated and necrotic areas developed at the sites of injection. As shown by chemical analysis of these areas, more than $\frac{3}{4}$ of the injected lead remained at the site of injection, either bound to the tissues or precipitated in the tissue fluids; and the lead depots so formed permitted a continuing slow absorption of lead into other parts of the body. All the animals so treated eventually died, the survival time varying inversely with the dosage as shown in table 1. Thus, after 5 daily doses of 240

TABLE 1

The toxicity in rabbits of lead acetate injected subcutaneously once daily for five days

DOSAGE LEAD ACETATE		NO. OF RABBITS	SURVIVAL TIME, DAYS AFTER LAST INJECTION			
Per injection	Total		Individual animals		Mean	Median
mg./kg.	mg./kg.					
240	1200	9	(3)*	33	26	26
			8	34		
			9	39		
			17	40		
			26			
160	800	10	(4)*	34	32	32
			18	34		
			23	40		
			23	41		
			29	43		
120	600	10	19	34	35	34
			22	44		
			29	45		
			34	46		
			34	46		

* Died during treatment.

mg./kg. per injection, the rabbits died in 3 to 40 days, the survival time averaging 26 days after the last injection.

A series of animals receiving the latter dosage of lead acetate was then treated with a 2 per cent solution of BAL in peanut oil, containing 4 per cent benzyl benzoate as a solubilizing agent. Injections of BAL at 5, 10 or 20 mg./kg. were begun one day after the last injection of lead acetate and were repeated every 3 hours to a total of 4 doses daily, and continued for 5 days. The total BAL dosage thus varied from 20 to 80 mg./kg. daily.

As shown in table 2, in this first experiment the administration of BAL, far from protecting the animals, significantly hastened their death. At the smallest dosages of BAL (5 mg./kg. per injection) the average survival time was reduced from 26 days in the untreated control group, to 15 days; and at BAL dosages of

20 mg./kg., the animals died 1 to 12 days after the last injection of lead, the mortality being greatest within the first four days. These findings are in full agreement with those of Braun, Lusky and Calvery (13).

In a second similar experiment, however, BAL did not accelerate death, although it still failed to exert a protective action. In this second experiment,

TABLE 2

The effect of BAL on the survival of rabbits previously given repeated subcutaneous injections of lead acetate

Animals were given 5 daily subcutaneous injections of 20 per cent lead acetate at a dosage of 240 mg./kg. per injection. Intramuscular treatment with 2 per cent BAL in peanut oil and benzyl benzoate, begun 1 day later, was repeated every 3 hours for 4 doses daily, and continued for 5 days. Animals dying within 5 days therefore received less than the total dosage indicated below.

DOSAGE BAL		NO. OF RABBITS	SURVIVAL TIME, DAYS AFTER LAST INJECTION OF LEAD ACETATE			
Per injection	Total		Individual animals		Mean	Median
mg./kg.	mg./kg.					
0	0	9	(3)*	33	26	26
			8	34		
			9	39		
			17	40		
			26			
5	100	10	2	10	15	8
			3	16		
			3	29		
			5	38		
			6	40		
10	200	10	4	10	12	8
			4	12		
			5	13		
			5	22		
			6	34		
20	400	10	1	3	3	3
			1	3		
			2	3		
			2	4		
			2	12		

* Died during treatment with lead acetate.

rabbits were given 240 mg./kg. of lead acetate daily for 5 days, followed by BAL at dosages of 10 mg./kg., repeated every 3 hours for 4 doses daily and continued for 5 days. Both the treated and control groups died at approximately the same time interval of 30 to 60 days. This second experiment was carried out during the winter months, while the first experiment was carried out during the hottest part of the summer. Temperature has been reported to affect the course of

lead poisoning in both animals and man (15), and the discrepant results in the two experiments may perhaps be referable to this factor. In any event, BAL clearly showed no protective action in either group. Possible explanations for this failure are discussed in a following section.

The effect of BAL on urinary lead excretion. To determine the effect of BAL on the urinary excretion of lead, rabbits were first injected with lead acetate (120 mg./kg. given subcutaneously once daily for 5 days). At varying intervals thereafter, the animals were given a single intramuscular injection of 2 per cent BAL in peanut oil and benzyl benzoate, at either 20 or 10 mg./kg. Two or three successive 2-hourly urine specimens were collected by bladder catheter-

TABLE 3

The effect of a single intramuscular injection of BAL on the rate of urinary excretion of lead

All animals received 5 daily subcutaneous injections of 20 per cent lead acetate at 120 mg./kg. per injection, to a total of 600 mg./kg. A single injection of BAL was given intramuscularly at varying intervals thereafter. Two-hourly urinary specimens were collected before and after BAL by bladder catheterization and irrigation.

RABBIT NO.	DOSE BAL SINGLE INJECTION	TIME OF ADMINIS- TRATION OF BAL (DAYS AFTER LAST INJECTION OF LEAD ACETATE	HOURLY EXCRETION OF LEAD IN MICROGRAMS						INCREASE IN RATE OF URINARY EXCRETION OF LEAD (REFERRED TO IMMEDIATE PRE-BAL LEVEL AS 100)		
			Before BAL			After BAL					
			6-4 hrs.	4-2 hrs.	2-0 hrs.	0-2 hrs.	2-4 hrs.	4-6 hrs.	1st 2 hrs.	2nd 2 hrs.	3rd 2 hrs.
	mg./kg.								%	%	%
A-5967	10	1	—	7.9	6.8	247	28.9	—	3630	425	—
A-4668	10	1	—	—	18.1	225	47.8	—	1240	260	—
A-6182	10	5	—	4.4	5.3	59.5	13.0	—	1120	245	—
A-6167	10	5	—	5.9	3.0	80.8	7.2	—	2690	240	—
A-5156	20	1	—	5.8	5.8	231	80.5	10.5	3980	1390	180
A-5833	20	1	16.4	8.8	8.4	96.0	61.5	10.8	1140	730	130
A-5920	20	9	—	5.1	7.4	137	64.2	19.3	1860	865	260
A-5920*	20	11	7.2	8.2	6.4	104	64.7	12.2	1630	1010	260

*NOTE: Rabbit A-5920 received a second injection of BAL 48 hours following the first injection.

ization and irrigation before and after the administration of BAL, and their lead content determined by the dicolorimetric dithizone method as described by Bambach (16).

The results of eight experiments, in which a single injection of BAL was given 1 to 11 days after the last injection of lead acetate, are summarized in table 3 and fig. 1. In fig. 1, the hourly urinary excretion of lead before and after the injection of BAL is plotted on a percentage basis, referred to the immediate pre-BAL rate as 100.

A single injection of BAL at 10 or 20 mg./kg., given 1, 5, 9 or 11 days after the last injection of lead acetate, was followed by an 11- to 40-fold increase in the urinary lead excretion. Within the time limits of this experiment, the interval between the last injection of lead and the following injection of BAL had no

effect on the magnitude of the excretion response to BAL. In view of the large lead depot in these animals (an average total of 800 mg. of lead, approximately $\frac{3}{4}$ of which remains at the site of injection¹), the continuing absorption from that depot, and the relatively scanty urinary excretion prior to the administration of BAL, this result is not surprising. The tissues clearly retain large amounts of

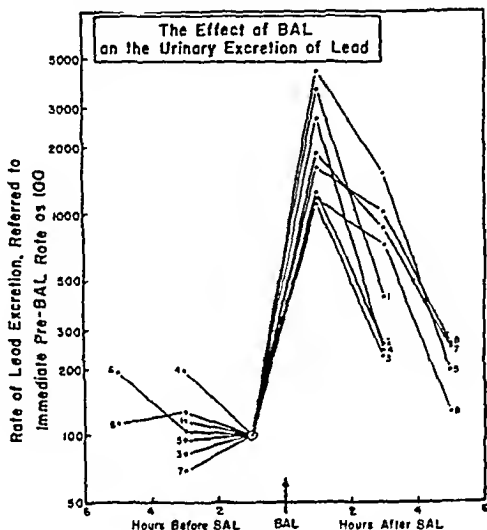


FIG. 1

All rabbits received 5 daily subcutaneous injections of 20 per cent lead acetate at 120 mg./kg. per injection. A single injection of a 2 per cent solution of BAL in peanut oil and benzyl benzoate was given intramuscularly at varying intervals thereafter. Two-hourly urinary specimens were collected by bladder catheterization and irrigation before and after BAL. Curves 1-4 represent the urinary response of 4 animals given a single injection of BAL at 10 mg./kg. per injection 1, 1, 5 and 5 days, respectively, after the last injection of lead acetate. Curves 5-8 represent experiments in which 3 animals were given an injection of BAL at 20 mg./kg. per injection, 1, 1, and 9 and 11 days, respectively, after the last injection of lead acetate. The hourly urinary excretion of lead before and after BAL has been plotted on a percentage basis, referred to the immediate pre-BAL rate as 100.

lead for a long period of time, and respond maximally to an injection of BAL whether that injection is given 1 or 11 days after the last injection of lead acetate.

¹ Skin and subcutaneous tissue taken from the sites of injection 6 days after the administration of lead acetate had a lead content estimated to represent more than three-fourths of the amount originally injected:

WGT. OF SAMPLE	APPROX. PER CENT OF TOTAL AREA INVOLVED	LEAD CONTENT	CALCD. TOTAL LEAD (ALL SITES)	TOTAL LEAD INJECTED	PER CENT INJECTED LEAD REMAINING AT SITE OF INJ.
gm.		mg.		mg.	
2.68	8	73.8	922	1160	79
2.12	12	120.0	1000	1160	86

As would be expected, the urinary response was more pronounced and more sustained after an injection of BAL at 20mg./kg. than after one of 10 mg./kg. After the larger dose, the rate of urinary excretion increased 11- to 40-fold for the first two hours, but continued at a high level for some time thereafter, so that in the second 2 hours it was still 7 to 14 times the pre-BAL rate. At BAL dosages of 10 mg./kg. the rate of urinary excretion increased 11- to 36-fold for the first 2 hours, but in the second 2 hours the urinary excretion was only 2 to 4 times the average pre-BAL rate.

It is important to note that, as in the case of arsenicals (7), the favorable effect of BAL injected at 5 to 20 mg./kg. is largely completed in 4 hours, reflecting its rapid destruction and elimination. A single injection of BAL at 20 mg./kg., the absolute dose averaging 60 mg., resulted in the excretion of 0.28 to 0.6 mg. of lead in the following four hours, over and above that which would have been excreted without BAL; and the injection of approximately 30 mg. of BAL (10 mg./kg.) resulted in the similar excretion of 0.13 to 0.52 mg. of lead, averaging 0.34.

The effect of multiple injections of BAL on the urinary excretion of lead. To determine the effect of repeated injections of BAL on the urinary lead excretion, 4 rabbits with a subcutaneous lead depot (120 mg./kg. of lead acetate daily for 5 days) were treated with BAL at 10 mg./kg. per injection over a 5-day period, the animals receiving 4, 2, 0, 4, 1 injections on successive days to a total of 11 injections. Two-hourly urine samples were collected before and after the administration of BAL. Because of the local trauma caused by repeated catheterization, it was not usually feasible to obtain more than a total of 3 to 5 specimens from a single animal. Despite the limited number of specimens, and the fact that they derived from different rabbits, the trend was nevertheless clear. As shown in table 4 and fig. 2, the magnitude of the lead-excretion response to BAL decreased regularly with each additional injection. With the first injection there was a 35-fold increase in the 2-hourly excretion of lead; this, however, decreased progressively, to the degree that the eleventh injection of BAL caused only a 2- to 3-fold increase in the 2-hourly excretion of lead.

In all, 11 successive injections of BAL caused the urinary excretion of a total of approximately 3.4 mg. of lead. The relatively small increase in urinary excretion after the last few injections of BAL indicates that this amount constituted almost the entire store of readily mobilizable lead in these animals. The site of deposition of this "labile" lead is not clear from the present experiments. It is probably not the subcutaneous depot itself, for the total amount mobilized by BAL represents only a minute fraction of that originally deposited and retained in the skin (cf. page 401). It will be of interest to determine whether, after the exhaustion of this "labile" lead by BAL, a similar readily mobilized store is again built up in the tissues by a continuing slow absorption from the subcutaneous depot. Under such circumstances, a repeated course of BAL would give the same excretion response as that indicated in fig. 1.

The effect of BAL on the intestinal excretion of lead. Because of the marked increase in the urinary excretion of lead caused by BAL, its effect on intestinal

excretion was also studied. Two animals were injected subcutaneously with lead acetate once daily for 5 days, at a dosage of 120 mg./kg. per injection. Three days after the last injection of lead acetate, the animals were treated with BAL at 10 mg./kg., repeated every 4 hours for 4 doses. Twenty-four hour fecal and urinary specimens were obtained before, during and after the administration of BAL. As shown in table 5, although BAL caused a 2- to 4-fold increase in the urinary lead excretion over the total 24-hour period, the fecal lead excretion was actually diminished. An associated decrease in fecal output may perhaps have obscured a possible effect of BAL; but it seems clear that it did not cause a marked acceleration in the fecal excretion of lead.

TABLE 4

The effect of multiple injections of BAL on the urinary excretion of lead in rabbits with a subcutaneous depot

All animals received 5 daily subcutaneous injections of 20 per cent lead acetate at 120 mg./kg per injection. Intramuscular treatment with 2 per cent BAL in peanut oil and benzyl benzoate was begun 1 day later at 10 mg /kg per injection, repeated every 4 hours for 4 doses the 1st day, 2 doses the 2nd day, 4 doses the 4th day and one dose the 5th day. Two hourly urinary specimens were collected by bladder catheterization and irrigation before and after several of the injections of BAL as indicated below

RABBIT NO	BAL INJECTION NO	HOURLY EXCRETION OF LEAD IN MICROGRAMS				INCREASE IN HOURLY EXCRETION OF LEAD (REFERRED TO PRE BAL LEVEL AS 100)	
		Before BAL		After BAL		1st 2 hrs	2nd 3 hrs
		4-2 hrs	2-0 hrs	0-2 hrs	2-4 hrs		
A 5967	1	7 9	6 8	247	28 9	3630	425
	2	—	—	173	45 1	2550	664
	5	—	16 4	94 0	12 1	574	74
A-4665	1	—	18 1	225	17 8	1240	264
	3	—	—	132	47 2	730	260
A 5787	11	26 0	12 8	42 9	15 3	335	119
A-5877	11	6 8	7 3	18 7	4 0	256	55

The effect of BAL on the histopathological changes produced by lead poisoning. Because BAL sometimes accelerates death in animals poisoned with lead acetate, an attempt was made to determine the effect of BAL on the histological changes produced by lead. One group of 6 animals received lead alone (5 daily subcutaneous injections of lead acetate at 180 mg./kg. per injection each); in a second similar group the same regime was followed by treatment with BAL in peanut oil and benzyl benzoate at 10 mg./kg., repeated 4 times daily at 3-hour intervals for 4 days; and a third group was given BAL in that same dosage without lead. Three animals in each group were killed at the completion of BAL treatment, and the others at varying intervals thereafter.

Animals killed immediately after the administration of lead showed no patho-

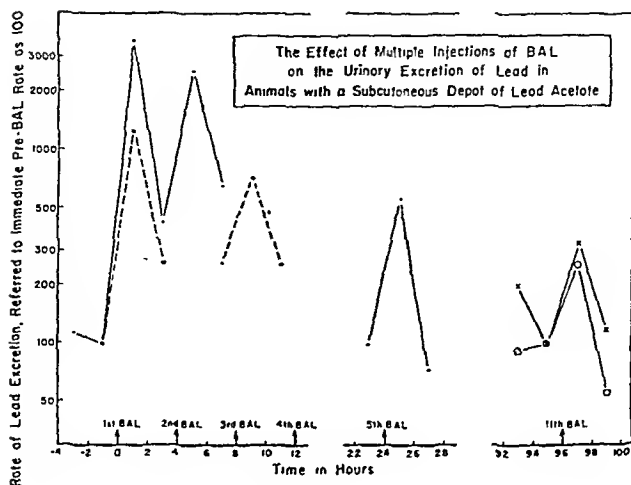


FIG. 2

All animals received 5 daily subcutaneous injections of 20 per cent lead acetate at 120 mg./kg. per injection. Intramuscular treatment with 2 per cent BAL in peanut oil and benzyl benzoate was begun 1 day later at 10 mg./kg. per injection, repeated every 4 hours for 4 doses on the first day, 2 doses on the second day, 4 doses on the fourth day and a single dose on the fifth day, to a total of 11 injections. Two-hourly urinary specimens were collected by bladder catheterization and irrigation. With one rabbit (curve —) specimens were collected before and after the first, second and fifth injections of BAL; with another (curve - - -) before and after the first and after the third injection; and with 2 others (curves: o—o, x—x) before and after the eleventh injection. Ordinates represent the rate of lead excretion referred to that of the immediate pre-BAL period as 100.

TABLE 5

The effect of BAL on the intestinal excretion of lead

Animals received 5 daily subcutaneous injections of 20 per cent lead acetate at a dose of 120 mg./kg. per injection, or a total of 600 mg./kg. Three days after the last injection of lead acetate a 2 per cent solution of BAL in peanut oil and benzyl benzoate was injected intramuscularly at 10 mg./kg., repeated every 4 hours for 4 doses. Twenty-four hour urinary and fecal collections were made one day prior to, during and after treatment with BAL.

RABBIT NO.	DAILY SPECIMEN NO.	TOTAL VOL. OF URINE	TOTAL URINARY LEAD	TOTAL WTGT. OF FECES	TOTAL FECAL LEAD
		cc.	mg.	gm.	mg.
A-7027	1	94.5	236	44.6	1990
	2 (BAL)	111	533	20.1	1180
	3	98.7	249	45.3	2780
A-7133	1	140	238	23.7	1170
	2 (BAL)	151	313	16.1	937
	3	136	298	16.8	714

logical changes. In those animals which survived the administration of lead acetate for 4 weeks or more, numerous intranuclear inclusion bodies as described

by Blackman (17) were seen in sections of the kidneys, with occasional similar inclusions in the liver. Except for occasional focal areas of neuronophagia, no lesions were seen in brain sections.

There was no evidence that the administration of BAL had any effect on the number or extent of these lesions, but it should be pointed out that BAL did not accelerate the death of the animals in this particular series.

The paucity of the pathologic changes caused by lead is perhaps not surprising in view of the scanty lesions often seen at autopsy in children dying of lead poisoning. The insignificant number and extent of these lesions may be out of all proportion to the severity of the symptoms and the fatal outcome.

TABLE 6

The acute toxicity in rabbits of lead acetate injected intravenously four times at three-hour intervals

DOSAGE		NO. OF RABBITS	SURVIVAL TIME IN DAYS		
Per injection	Total		Individual animals		Median
mg./kg.	mg./kg.				
24	96	5	1	1	1
			1	6	
			1		
16	64	5	2	6	3
			2	7	
			3		
12	48	9	(1)*	1	3
			1	3	
			1	4	
8	32	4		4	18
				14	
6	24	5	2	3	2.5
			2	5	
			(1)*	10	6
			6	14	
			6		

* Died before completion of injection.

B. THE EFFECT OF BAL IN ACUTE LEAD POISONING IN RABBITS. *The effect of BAL on survival.* A series of rabbits was injected intravenously with 3 per cent lead acetate, repeated four times at 3-hourly intervals at dosages per injection ranging from 6 to 24 mg./kg. As seen in table 6, all the animals died within 1 to 18 days.

A series of rabbits similarly injected at 12 mg./kg. per injection was then treated with BAL in peanut oil and benzyl benzoate, beginning 8 hours after the last injection of lead acetate. The BAL dosage, varying from 5 to 20 mg./kg., was repeated at 4-hour intervals 5 times on the first day, and 3 times on the second day. As shown in table 7, BAL so administered had no effect on survival, the animals dying in approximately the same time as the untreated controls.

The effect of BAL on the urinary excretion of lead. In these acutely poisoned animals, in which large amounts of lead were excreted in the urine, one might have expected BAL to have little or no additional effect². However, as shown in table 8, a single injection of BAL at 10 mg./kg. caused a 3- to 7-fold increase in the urinary excretion of lead for a 2-hour period. Two of the three animals under test became moribund during the latter half of the experiment, and their urinary lead-excretion decreased correspondingly.

TABLE 7

The effect of BAL on the survival of rabbits with acute lead poisoning

Animals were injected intravenously 4 times at 3-hour intervals with 3 per cent lead acetate, at a dosage of 12 mg./kg. per injection (total of 48 mg./kg.). Intramuscular treatment with 2 per cent BAL in peanut oil and benzyl benzoate was begun 8 hours later at dosages indicated below, and was repeated every 4 hours for 5 doses on the first day and for 3 doses on the second day. Animals dying within less than two days received less than the total of 8 injections of BAL.

DOSAGE BAL		NO. OF RABBITS	SURVIVAL TIME, DAYS AFTER LAST INJECTION OF LEAD ACETATE
Per injection mg./kg.	Total mg./kg.		
20	160	4	1
			2
			2
			8
10	80	4	1
			1
			2
			4
5	40	4	1
			1
			1
			6
0	0	4	1
			1
			4
			4

Histopathological findings in animals acutely poisoned with lead and receiving BAL. Six of the rabbits which died following the intravenous injection of lead acetate were immediately autopsied and sectioned. The only microscopic abnormality found was the presence in three of the animals of hemoglobin casts in the renal tubules. In one instance, the kidneys were massively involved; the

² Thus, BAL has a relatively slight effect on the excretion of mapharsen (3-amino-4-hydroxyphenyl arsenoxide), which is normally excreted at a rapid rate; but it has a striking effect on the excretion of phenyl arsenoxide, which is otherwise excreted very slowly (7)

others showed involvement to a lesser degree. The intravenous injection of lead acetate apparently had produced intravascular hemolysis, occasionally evidenced by hemoglobinuria during life. Two of four rabbits acutely poisoned with lead and receiving BAL showed similar casts in the renal tubules on microscopic examination of tissue sections. No other lesions were seen in either group.

C. THE PREPARATION AND TOXICITY OF THE LEAD-BAL COMPLEX. When a solution of lead acetate is added to an aqueous solution of BAL, a copious flocculent yellow precipitate is formed, in whatever proportion the reactants are mixed.

TABLE 8

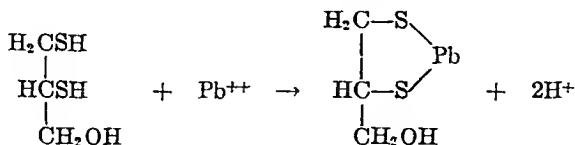
The effect of BAL on the urinary excretion of animals with acute lead poisoning

All animals were injected intravenously every 3 hours for 4 doses with 0.5 per cent lead acetate in saline or 3 per cent lead acetate in aqueous solution, at 6 mg./kg. per injection to a total of 24 mg./kg. Treatment with BAL at 10 mg./kg. was begun 8 hours after the last injection of lead acetate and repeated every 4 hours for 4 doses. Pre-BAL and post-BAL urinary specimens were obtained as indicated below.

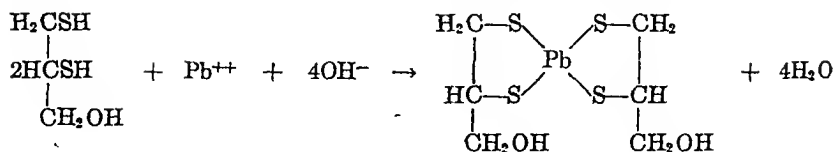
RABBIT NO.	BAL INJECTION NO.	HOURLY EXCRETION OF LEAD					INCREASE IN URINARY EXCRETION OF LEAD CAUSED BY BAL (REFERRED TO IMMEDIATE PRE-BAL LEVEL AS 100)	
		Before BAL			After BAL		1st 2 hrs.	2nd 2 hrs.
		8-6 hrs.	6-2 hrs.	2-0 hrs.	0-2 hrs.	2-4 hrs.		
		mg.	mg.	mg.	mg.	mg.	%	%
A-6629	1	18.6	37.3	27.7	105	35.9	380	130
	4	—	—	—	84	—	303	—
A-6637	1	8.3	21.1	18.1	128.8	—	711	—
	4	—	—	—	*17.3	*10.4	*95	*58
A-6776	1	—	—	—	91.4	52.8	266	154
	2	—	—	—	*32.3	*6.3	*95	*18

* Animals were moribund at this time, and urinary flow was markedly reduced.

A sample of the washed and dried precipitate contained 60.1 per cent lead, indicating that the 2 compounds had reacted mole for mole:



The freshly precipitated lead-BAL complex was insoluble in water, in acid and alkali, and in the commoner organic solvents. It was, however, soluble in 1N NaOH which had been previously saturated with BAL. This suggests that under these conditions lead may form a soluble complex with BAL, in which two moles of BAL enter into combination with one mole of lead:



For intravenous injections, the lead-BAL complex was dissolved in IN NaOH saturated with BAL, and the solution was then adjusted to pH 8-8.5 with acetic acid. At this pH the solution of lead-BAL assumed a yellowish colloidal cast; at a more acid reaction the lead-BAL complex precipitated. The toxicity of

TABLE 9

The relative toxicity of the Lead-BAL complex and lead acetate

Rabbits were injected intravenously with 0.5 per cent Lead-BAL (60.1% Pb) complex in saline (pH = 8.6) every 3 hours for 4 doses. Control rabbits were injected similarly with 0.5 per cent lead acetate (54.1% Pb) in saline. All animals living and well one month after treatment were considered as survivors.

COMPOUND	DOSAGE			NO. OF RABBITS	DIED	SURVIVED	LD ₅₀		LD ₅₀	
	Per inj.	Total					Compound	Lead	Compound	Lead
		com- pound	lead							
	mg./kg.	mg./kg.	mg./kg.				mg./kg.	mg./kg.	mg./kg.	mg./kg.
Lead-BAL	24	96	57.7	3	3	0	50	30	63	38
	16	64	38.5	6	6	0				
	12	48	28.8	6	2	4				
	8	32	19.2	6	1	5				
	6	24	14.4	6	0	6				
	4	16	9.6	2	0	2				
Lead acetate	24	96	52.4	3	3	0	20	16	64	35
	16	64	34.9	6	5	1				
	12	48	26.2	6	5	1				
	8	32	17.5	6	5	1				
	6	24	13.1	6	2	4				
	4	16	8.7	2	0	2				

the lead-BAL complex on repeated intravenous injection was then determined, in comparison with that of lead acetate similarly injected as a 0.5 per cent solution in 0.85 per cent NaCl.

In table 9, animals living and well one month after the injection are listed as survivors. As there shown, although the LD₅₀ of the lead-BAL complex was twice that of lead acetate (30 and 16 mg./kg., respectively, expressed in terms of milligrams of lead), the LD₅₀ values were essentially the same (35 and 38 mg./kg. of lead, respectively). The lead-BAL complex is thus almost as toxic as lead acetate itself, despite the probably low dissociation constant of the soluble complex used for injection.

The high toxicity of the lead-BAL complex may explain, at least in part, the

fact that in some experiments (cf. (13) and page 398) the administration of BAL to lead-poisoned animals actually accelerated their death. The mobilization of large amounts of lead from tissues, with the formation of a soluble and toxic lead-BAL complex, may have permitted that complex to act on organs more vulnerable to the toxic effects of lead, or more vital to the host, than were the original sites of deposition.

DISCUSSION. In the present experiments, it has been shown that BAL can dissociate lead from its combination with tissues. This was manifested by the striking increase in the rate of urinary lead excretion following the administration of BAL to rabbits poisoned with lead acetate. Nevertheless, BAL afforded no protection in rabbits with either acute (intravenous) or subacute (subcutaneous) lead poisoning. There are several possible explanations for this failure. Relatively large quantities of lead acetate are necessary to cause the death of rabbits. Most of this is firmly bound by the tissues, and only a small proportion is mobilized even by multiple injections of BAL. The amount eliminated from the body under the influence of BAL is therefore too small to affect the outcome.

A second reason for the failure of BAL to protect animals against lead poisoning may be the fact that the lead which is mobilized by BAL forms a lead-BAL complex which is almost as toxic as the lead salt itself. This probably explains the fact that in some experiments intensive BAL therapy actually accelerated the toxic effects of lead. In such cases the lead which has been mobilized from the tissues to form a lead-BAL complex may then act on other organs either more vulnerable to the toxic effects of lead, or more vital to the bodily economy, than were the tissues in which the lead was originally deposited.

Whatever the correct explanation, it is clear that under the conditions of the present experiments, BAL had no demonstrable protective action, and at times actually accelerated the toxic effects of lead acetate in rabbits. The data offer no reason to believe that BAL would prove of value in the treatment of lead poisoning in man. Nevertheless, the experimental lead poisoning produced in rabbits by either the subcutaneous or intravenous injection of lead acetate differs so materially from that seen in man, both with respect to the route of administration, the chemical form of the lead, the chronicity of the intoxication, and the organs affected, that the results obtained with BAL in these experimental animals have no necessary significance with respect to its possible therapeutic activity in man. Despite the present complete failure of BAL to detoxify lead in rabbits, its effect on the urinary excretion of lead was so striking as perhaps to justify its cautious therapeutic trial in human cases.

SUMMARY

1. Five consecutive daily subcutaneous injections of lead acetate at a dosage of 240 mg. of compound per kg. per injection regularly caused the death of rabbits within 3 to 40 days after the last injection. The intravenous injection of the same salt at a dosage of 12 mg./kg. per injection, repeated every 3 hours for 4 doses, produced an acute poisoning from which the animals died in 1 to 18 days, averaging 3 days.

2. The intramuscular administration of BAL in peanut oil and benzyl benzoate, at individual dosages of 5 to 20 mg./kg., and repeated every 4 hours, failed to protect animals poisoned with lead acetate by either route of administration. In one group of animals with subacute (subcutaneous) lead poisoning, the animals treated with BAL died significantly faster than did the corresponding untreated controls.

3. The administration of BAL caused a marked increase in the urinary excretion of lead. For 2 hours after a single injection of BAL at 20 mg./kg., the urinary excretion of lead increased 11- to 40-fold in animals with a subcutaneous depot, and 3- to 7-fold in animals injected intravenously. This favorable effect on the excretion of lead lasted for approximately four hours after a single injection of BAL. The magnitude of the excretion response diminished with each additional injection of BAL, suggesting that only a small fraction of the lead injected could be dissociated by BAL from its combination with tissues.

4. The failure of BAL to protect these animals may be in part due to the fact that it mobilizes only a small fraction of the total body store of lead, and in part to the fact that the lead-BAL complex proved almost as toxic as the lead salt itself on intravenous injection.

5. The only pathological changes produced by subcutaneous injections of lead acetate were occasional intranuclear inclusions in the renal epithelium and liver parenchyma, seen only in those animals which survived an eventually lethal dose for 3 weeks or more. Treatment with BAL had no demonstrable effect on the development of these inclusions.

6. The implications with respect to the possible use of BAL in the treatment of lead poisoning in man are discussed in the text.

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HISTAMINE SHOCK IN MICE SENSITIZED WITH HEMOPHILUS PERTUSSIS VACCINE

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In work on hypersensitivity of laboratory animals to *Hemophilus pertussis* antigens, we observed that mice injected with a vaccine of this organism became sensitive to histamine, a result which seemed worthy of further investigation.

Accordingly, mice were sensitized with massive doses of partially detoxified *H. pertussis* vaccine as described in previous publications (1-3), or in some cases with standard Kendrick vaccine (4). Following the sensitizing injection, sensitivity was tested by injecting the mice intraperitoneally with histamine diphosphate. Hypersensitivity to histamine developed by the fifth day after the vaccine injection, and persisted at a high level for 11-12 days. Death of the sensitized mice occurred 5-30 minutes after the histamine injection.

In one experiment, mice were sensitized by intraperitoneal injection of 75 billion cells of *H. pertussis* (defatted Kendrick vaccine) and injected intraperitoneally 5 days later with different amounts of histamine diphosphate. See table 1.

Table 1 shows that under the conditions of this experiment, 2 mg. of histamine diphosphate are about as toxic to sensitized mice as 50 mg. are to normal mice.

The sensitivity to histamine after injection of varying amounts of *H. pertussis* vaccine is shown in table 2.

Mice injected with 20 billion or more cells (0.05 mg. or more of total N) showed very high sensitivity to histamine. Smaller injections (5 billion cells, 0.014 mg. total N) produced a lesser but very distinct hypersensitivity.

In contrast to the results with *H. pertussis* vaccine, mice injected with typhoid vaccine or with normal horse serum, and injected with histamine on the fifth day (or later) showed no hypersensitivity. See table 3.

Table 3 shows that (under the conditions of our experiments) neither typhoid vaccine nor normal horse serum produced a sensitivity to histamine, even when the amount of histamine injected as a shocking dose was 10 times that used in testing sensitization by *H. pertussis* vaccine.

It was considered of interest to determine whether the hypersensitivity to histamine resulting from sensitization with *H. pertussis* could be counteracted by antihistamine agents. See table 4.

Table 4 shows that the antihistamine drug Bromothen, N,N-dimethyl-N'-(2-pyridyl)-N'-(5-bromo-2-thenyl)-ethylenediamine hydrochloride (5), in adequate dosage protects hypersensitive mice against histamine.

SUMMARY

Mayer and Brousseau (6) and Perry and Darsie, Jr. (7) recently reported some very comprehensive studies on anaphylaxis in mice, but the significance of

TABLE 1

Action of histamine diphosphate on normal mice and upon mice sensitized with 75 billion cells of *H. pertussis* 5 days previously*

HISTAMINE DIPHOSPHATE	NORMAL MICE	HISTAMINE DIPHOSPHATE	SENSITIZED MICE
mg.		mg.	
100	0/5	5	0/8
50	1/6	2	3/15
20	10/10	1	3/8
		0.5	4/6

* Fractions represent number of mice surviving divided by total number used.

TABLE 2

Sensitivity of mice injected with different amounts of *H. pertussis* vaccine and given 2 mgm. of histamine diphosphate intraperitoneally on the fifth day

	BILLIONS OF CELLS PER MOUSE			
	75	20	5	1
Mg. of total N in the vaccine ..	0.2	0.05	0.014	0.003
Survivals	4/28	1/9	5/12	11/12

TABLE 3

Tolerance to histamine of mice injected with typhoid vaccine or with normal horse serum

	TYPHOID VACCINE (15 BILLION CELLS)	NORMAL HORSE SERUM	
		(2 cc. repeated at 24 hrs.)	(0.2 cc. repeated at 24 hrs.)
Total N injected, Mg.	0.077	23.4	2.34
Survivals (after injection of 20 mg. of histamine diphosphate)	8/8	7/8	7/8

TABLE 4

Antihistamine action of Bromothen in mice sensitized (to histamine) by (means of) *H. pertussis* vaccine

BROMOTHEN PER MOUSE (INJECTED 15 MINUTES BEFORE THE HISTAMINE)	HISTAMINE DIPHOSPHATE PER SENSITIZED MOUSE	
	5 mg. (2.5 lethal doses)	10 mg. (5 lethal doses)
mg.		
0.05	3/4	1/2
0.1	11/13	2/10
0.3	10/10	5/10
0.5		9/10

histamine was not determined. However, in our own experiments it was found that *H. pertussis* vaccine injected into mice produced a hypersensitivity to histamine, which could be counteracted by the antihistamine agent Bromothen.

It has not yet been determined whether the injection of *H. pertussis* vaccine into man can produce similar effects.

Acknowledgment: Thanks are due to Dr. Austin Joyner for his interest in the work and for his many useful suggestions; also to Dr. Guy W. Clark for his help in the preparation of this manuscript.

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ACUTE VASCULAR TOLERANCE TO MORPHINE, ISONIPECAINE (DEMEROL), AND METHADON (AMIDONE) IN THE DOG¹

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The term "acute" tolerance was suggested by Schmidt and Livingston in 1928 (1) to represent the gradual disappearance of a characteristic reaction to morphine and the phenomenon was first described as a changing response to the circulatory effect of the drug. These workers found that intravenous injection of 1 mg. per kg. or more of morphine into dogs or cats produced a sudden fall in blood pressure followed by gradual recovery occurring over a prolonged period. Subsequent injections gave diminishing responses until finally no depressor reaction followed doses as high as 5 mg. per kg. This depressor effect, they pointed out, was largely due to a direct dilator action on peripheral vessels.

In subsequent papers (2, 3) Schmidt and Livingston offered evidence of the occurrence of acute tolerance in other tissues (cerebral cells, the vasomotor and respiratory centers) than the blood vessels. This was not true in the case of the intestine. In addition, acute tolerance was shown to develop not only to morphine but also to codeine, heroin, and pseudomorphine. When acute tolerance was established to any one of these compounds, injection of any other failed to produce its circulatory effect, i.e. cross tolerance was present. Tatum, Seevers and Collins (4) have demonstrated the ability of the dog to develop acute vascular tolerance to thebaine, and subsequent cross tolerance to the depressant drugs in the morphine series. Seevers (5) has also found that acute tolerance along with cross tolerance to the depressant members of the series occur with apocodeine, apomorphine and certain convulsant analogues.

In these experiments the ability of the dog to develop acute vascular tolerance to morphine and two newer analgesics, isonipecaine (Demerol) and methadon (Amidone) was investigated with the hope that such a procedure might be of some aid in screening the potential tolerance and addiction capacity of new analgesic compounds. The presence or absence of cross tolerance was also tested.

METHODS Two types of experiments employing dogs were used in this investigation: 1) Animals anesthetized with 15 mg. per kg. of Pentothal Sodium and 250 mg. per kg. of sodium barbital intravenously followed by exposure and cannulation of the carotid artery for recording blood pressure, 2) Unanesthetized animals in which blood pressure was recorded from the femoral artery following exposure and cannulation under local anesthesia. The drugs were employed in the following dosage: morphine sulfate—2 or 4 mg. per kg., Demerol hydrochloride—5 mg. per kg., Amidone hydrochloride—2 mg. per kg. All injections were made into the femoral vein. Blood pressure was allowed to return to approximately the same level as was present prior to injection before any further drug was administered.

¹ Supported by a grant from Parke, Davis & Company, Detroit, Michigan

RESULTS. In the anesthetized dog, all three compounds, when injected intravenously, produce an acute fall in blood pressure. Characteristic responses are portrayed graphically in fig. 1. The period of hypotension is characterized by the abrupt transient drop in arterial pressure with partial recovery usually occurring in 2 to 5 minutes, merging into a less severe but more prolonged period of hypotension from which recovery gradually occurs in 30 to 180 minutes. In the unanesthetized dog morphine produces the same response qualitatively and quantitatively. The duration of the hypotensive period in the unanesthetized animal is slightly shorter with Demerol and much shorter with Amidone than in the anesthetized animal.

Morphine sulfate in successive doses of 2 or 4 mg. per kg. produces decreasing hypotensive responses and finally complete vascular tolerance as described by Schmidt and Livingston (fig. '1). The unanesthetized animal behaves similarly

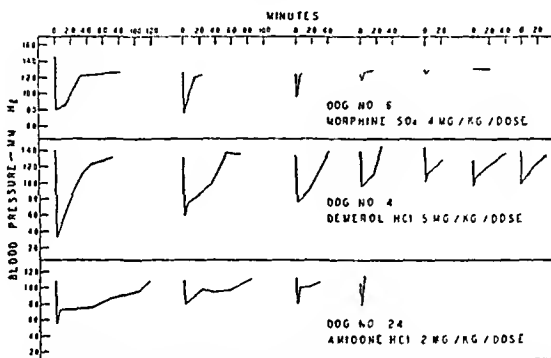


FIG. 1. THE EFFECT OF REPEATED INTRAVENOUS INJECTIONS OF MORPHINE, DEMEROL AND AMIDONE ON THE BLOOD PRESSURE OF THE DOG

The hypotensive response to repeated injections of Demerol (fig. 1) gradually diminishes for the first few doses and then remains constant, only partial tolerance being obtained. Some dogs, in the absence of anesthesia, develop complete tolerance to Demerol, and others show the same response as in the presence of anesthesia.

The anesthetized dog develops little or no tolerance to the abrupt transient fall in arterial pressure produced by the intravenous injection of Amidone, but after the first two doses of the drug, the animal recovers rapidly from the acute blood pressure drop, apparently having developed a tolerance to the prolonged, less severe effects of the compound, but not to its acute depressor action. The same type of response to repeated injections of Amidone is seen in the unanesthetized as in the anesthetized animal.

Administration of 5 mg. per kg. of Demerol intravenously to anesthetized dogs previously rendered tolerant to morphine produces hypotension of less degree and shorter duration than when given to animals non-tolerant to morphine (fig. 2). This response does not decrease significantly with succeeding

doses. There is produced a partial cross tolerance which is of the same degree as that following several injections of Demerol. Similar results are obtained in the unanesthetized animal. If Amidone is injected intravenously into an an-

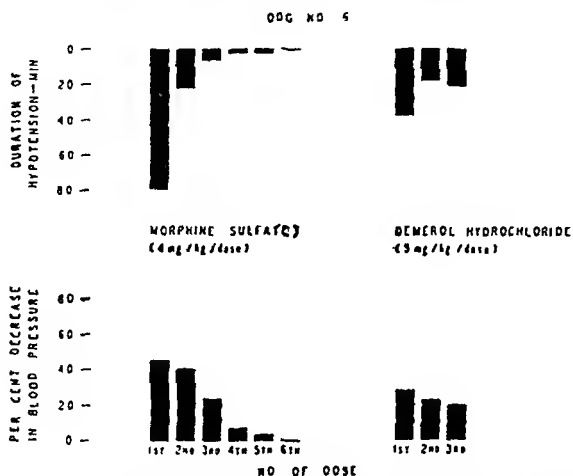


FIG. 2. THE EFFECT OF INTRAVENOUS DEMEROL ON THE BLOOD PRESSURE OF THE DOG FOLLOWING THE DEVELOPMENT OF ACUTE TOLERANCE TO MORPHINE

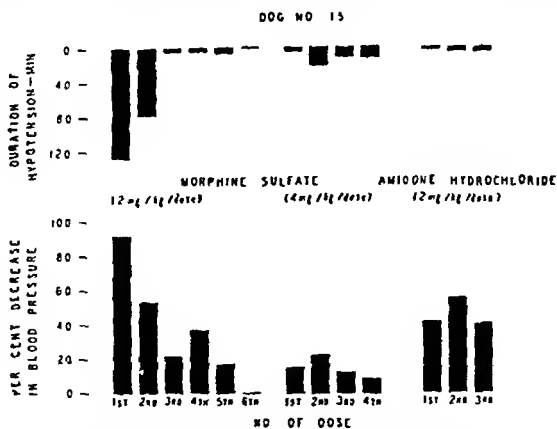


FIG. 3. THE EFFECT OF INTRAVENOUS AMIDONE ON THE BLOOD PRESSURE OF THE DOG FOLLOWING THE DEVELOPMENT OF ACUTE TOLERANCE TO MORPHINE

esthetized dog which is acutely tolerant to morphine, the same blood pressure response occurs as is obtained following two administrations of Amidone to an animal which had received no morphine previously (fig. 3). There is an acute fall in blood pressure which is of short duration. Tolerance to morphine confers

tolerance to the prolonged, less severe hypotensive period which ordinarily follows intravenous administration of Amidone. The same type of cross tolerance is seen in the unanesthetized dog.

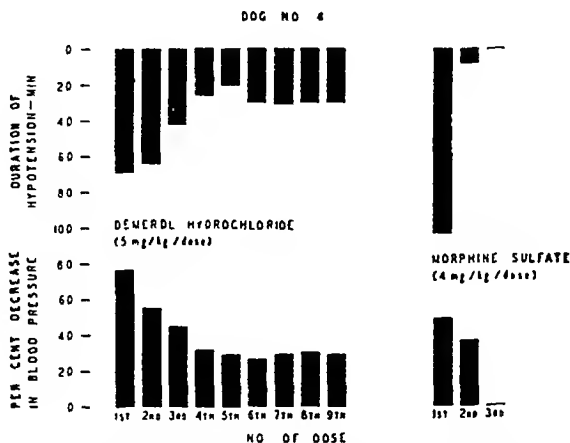


FIG. 4. THE EFFECT OF INTRAVENOUS MORPHINE ON THE BLOOD PRESSURE OF THE DOG FOLLOWING REPEATED DOSES OF DEMEROL

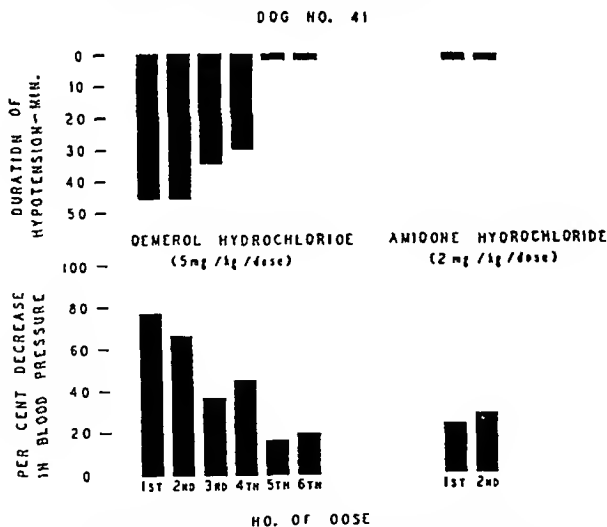


FIG. 5. THE EFFECT OF INTRAVENOUS AMIDONE ON THE BLOOD PRESSURE OF THE DOG FOLLOWING REPEATED DOSES OF DEMEROL

Repeated doses of Demerol in the anesthetized dog produce no tolerance to morphine (fig. 4). However, in the unanesthetized animal, Demerol confers partial to complete cross tolerance to morphine. After several injections of

Demerol, Amidone produces the same blood pressure response as is seen when it is given following development of acute tolerance to morphine (fig. 5).

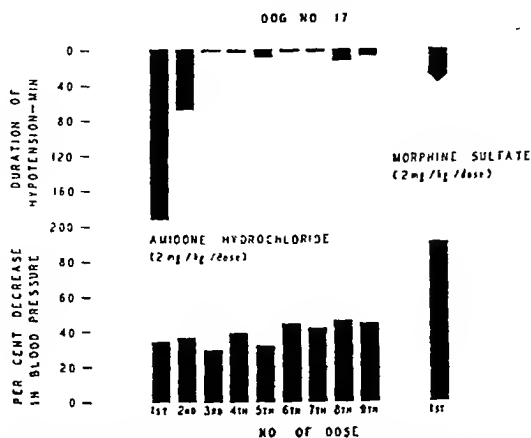


FIG. 6. THE EFFECT OF INTRAVENOUS MORPHINE ON THE BLOOD PRESSURE OF THE DOG FOLLOWING REPEATED DOSES OF AMIDONE

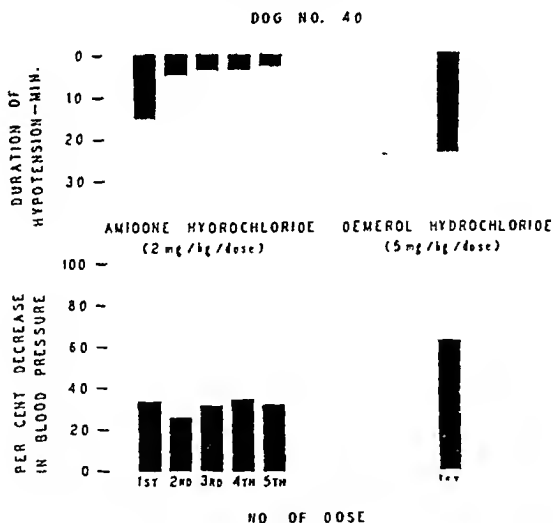


FIG. 7. THE EFFECT OF INTRAVENOUS DEMEROL ON THE BLOOD PRESSURE OF THE DOG FOLLOWING REPEATED DOSES OF AMIDONE

Repeated injections of Amidone, in the anesthetized or unanesthetized animal, results in no cross tolerance to morphine or Demerol (figs. 6 and 7).

DISCUSSION. In general, tolerance develops more rapidly in the absence of

anesthesia. In addition, the unanesthetized dog may develop complete tolerance to a compound which shows no potentiality in producing complete tolerance in the anesthetized dog. It would appear that anesthesia interferes in some way with the compensatory mechanisms involved in this tolerance reaction.

It is interesting to note the parallelism between the development of acute vascular tolerance in dogs and addiction and tolerance in man and the monkey. Humans have been shown to develop a high degree of tolerance to morphine, a lesser degree of tolerance to Demerol (6), and recent work by Woods, Wyn-gaarden and SeEVERS (7) indicates that monkeys show no tolerance to the depressant effects of Amidone. Himmelsbach also has shown that Demerol will relieve the withdrawal symptoms of morphine addicts (6), indicating some degree of cross tolerance. These observations parallel closely the acute tolerance experiments in dogs.

On the other hand, Wikler and Frank (8) have demonstrated the development of tolerance to the sedative, analgesic and hypothermic effects of Amidone in dogs. Isbell *et al.* (9) have presented evidence that Amidone will substitute quite adequately for morphine in human addicts. Eddy (10) has also demonstrated the development of tolerance to the analgesic action of Amidone in man. The manner in which vascular tolerance fits into the picture of tolerance as ordinarily conceived and the role it may play in the production of addiction is not clear at the present time although the parallelism suggests it may be a factor.

SUMMARY

In the anesthetized dog intravenous administration of morphine, Demerol or Amidone results in an acute fall in blood pressure with gradual recovery occurring over a variable period of time. Repeated injection of morphine (2 or 4 mg. per kg.) results in complete vascular tolerance. Only partial acute tolerance develops to Demerol (5 mg. per kg.) administered similarly. The acute drop in blood pressure and rapid recovery, without the succeeding prolonged period of hypotension occurring after two doses of Amidone demonstrates the development of tolerance to the prolonged depressor effect of this drug.

Tolerance to morphine confers only partial tolerance to Demerol and no tolerance to the acute hypotensive effect of Amidone. Repeated injection of Demerol produces no tolerance to morphine, no tolerance to the acute fall in blood pressure produced by Amidone, but tolerance to the prolonged period of hypotension occurring after a single dose of this latter drug.

Repeated administration of Amidone results in no cross tolerance to morphine or Demerol.

Anesthesia interferes with the compensatory mechanisms involved in the development of acute tolerance.

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INHIBITION OF PANCREATIC VACUOLIZATION

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Vacuolization of the exocrine pancreas was first reported in 1895 by Mouret, who noted vacuoles after the injection of pilocarpine (1). Babkin (2) produced them in 1909 by electrical stimulation of the vagus and LeBlond and Sergeeva (3) produced them by thyroxine and acetylcholine used separately in 1944. The latter investigators failed to find fat in the vacuoles and concluded that the vacuolization was due to autodigestion of the pancreatic acini by intraglandular activation of enzymes from vagotonic stimulation. These three papers seem to be the only ones in the literature dealing with the subject. This paper confirms previous findings, presents additional data on the physiological character of these vacuoles and describes a modification in technique. Since acetylcholine was the cause for this vacuolization, its antagonism by atropine was investigated to determine whether the vacuolization is mediated through the muscarinic effect of acetylcholine, i.e., direct stimulation of the effector organ (receptive substance of Langley).

TECHNIQUE. The animals used were white rats of both sexes varying in weight from 150 grams to 330 grams. Food was removed from the cage 2 hours before each experiment; water was allowed. All drugs were injected subcutaneously between the scapulae. The salts employed were acetylcholine bromide, atropine sulfate, and pilocarpine hydrochloride. All were administered on a pre determined body-weight basis.

Part I. Acetylcholine in a dose of 0.5 mg per 100 grams body weight was injected at intervals of 20 to 30 minutes for a period of 3 hours and 40 minutes. Nine animals were used. Previous to establishing the above routine, 0.5 mgm acetylcholine had been given, with 0.1 mgm physostigmine, for each 100 grams of body weight, to 7 rats, at intervals of 1 hour, according to the procedure of LeBlond and Sergeeva (3). These workers used a relatively large dose, namely 1 mgm of acetylcholine at 1-hour intervals for 3 doses. By utilizing acetylcholine alone, however, a more marked effect was produced and alterations by other agents could be more easily determined. Furthermore, the dose of acetylcholine and physostigmine employed by LeBlond and Sergeeva was fatal to rats used in the present experiment, which weighed 150 to 330 grams, whereas rats used by LeBlond and Sergeeva weighed 70 to 90 grams.

Part II. This experiment was identical with that described in Part I except that a single injection of atropine was given 1 hour before starting the acetylcholine routine. The dose of atropine ranged from as little as 0.1 mgm to 5.0 mgm per 100 grams of body weight. Eight animals were used. This same dose was used in 5 animals given the acetylcholine-physostigmine mixture mentioned in Part I.

Part III. Since each of the 3 animals used in this experiment weighed 160 grams, a standard dose of 5 mgm of pilocarpine was injected at 30 minute intervals for 8 doses.

Part IV. This experiment was identical to Part III except that 0.1 mgm to 7 mgm of atropine was given 1 hour before starting the pilocarpine routine. Six animals were used.

A control experiment was run using atropine alone in 5 rats to determine whether any detectable histological effects could be obtained. All animals were sacrificed by decapitation 20 minutes after the last injection. Specimens were taken of all parts of the gland from the portion adjoining the duodenum and pylorus to the portion adherent to the spleen.

The sections were stained in a routine manner with hematoxylin-eosin. In addition, several slides were treated with Sudan III fat stain. All sections were prepared by the author except for the fat stains and a few of the routine slides.

RESULTS. *Part I.* Widespread vacuolization was seen in pancreases of all animals treated with acetylcholine alone in the dose of 0.5 mgm. per 100 grams of body weight (see figures 2 and 3). Smaller doses produced less intense effects.



FIG. 1 (top). NORMAL PANCREAS. 100X

FIG. 2 (bottom). PANCREAS FROM ANIMAL TREATED WITH ACETYLCHOLINE, SHOWING VACUOLIZATION. 100X

On examination of the slides at 100X it appears that the vacuoles are grouped around the islets of Langerhans, but this has not yet been definitely established. A more marked degree of vacuolization was produced by acetylcholine alone without physostigmine.

Part II. There was no more vacuolization in the group treated with moderate doses of atropine than is found in the pancreases of untreated animals (see

figure 4). With the standard dose of acetylcholine used (0.5 mgm. per 100 grams of body weight) the barest evidence of vacuolization occurred with 0.1 mgm. of atropine per 100 grams of body weight. When the dose of acetylcholine was doubled the barest evidence of vacuolization occurred at 0.5 mgm. of atropine per 100 grams of body weight.

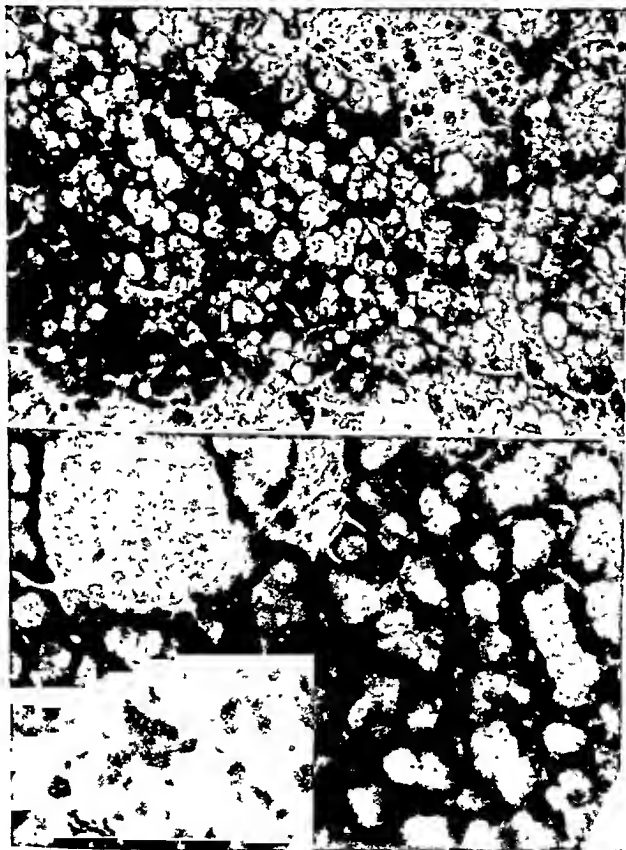


FIG. 3 (top). PANCREAS FROM ANIMAL TREATED WITH ACETYLCHOLINE. 440X
(HIGH MAGNIFICATION OF FIGURE 2)

FIG. 4 (bottom). PANCREAS FROM ANIMAL TREATED WITH BOTH ATROPINE AND ACETYLCHOLINE, SHOWING LACK OF VACUOLATION. 440X

Part III. Widespread vacuolization was seen in all pancreas sections obtained from animals treated with pilocarpine alone and this appeared to be identical with that caused by acetylcholine.

Part IV. No vacuolization was seen in the pancreases of rats treated with both pilocarpine and atropine, until the dose of atropine was lowered to 0.5 mgm. per 100 grams of body weight.

No fat could be detected in any of the vacuoles and no histological changes could be detected in pancreases treated by atropine alone.

DISCUSSION. By blocking the muscarinic effect of acetylcholine and of pilocarpine and thereby preventing the production of pancreatic vacuoles, we have shown that the site of action responsible for the vacuolating effect probably is the receptive substance postulated by Langley.

Preliminary experiments seem to indicate that epinephrine, also, inhibits the formation of vacuoles. Experiments dealing with this possibility are now in progress.

That increasing doses of acetylcholine require increasing amounts of atropine was to be expected since "within certain limits the antagonism between atropine and pilocarpine or acetylcholine is mutual and reversible" (4).

A less marked degree of vacuolization was seen following injections of physostigmine and acetylcholine than with acetylcholine alone. This is probably due to the fact that the mixture is often lethal, so that repeated injections of acetylcholine could not be made.

Vacuoles appear in the normal pancreas, but they are very small and are found only with careful searching, so that there can be no doubt concerning the relation of the vacuoles to the administration of acetylcholine.

CONCLUSIONS

1. Several findings of previous investigators were confirmed, as follows:
 - a. parenteral administration of massive doses of acetylcholine produces a vacuolization of the exocrine pancreas.
 - b. parenteral administration of pilocarpine produces the same vacuolization.
 - c. these vacuoles do not contain fat.
2. Atropine prevents the vacuolization produced by either acetylcholine or pilocarpine.
3. No histological changes are produced in the pancreas by moderate doses of atropine.

I wish to express my deepest appreciation to Professor C. H. Thiennes for making this project possible and for his kind assistance and advice. I wish, also, to express my gratitude to S. Bernick for his advice in the preparation of the microscopical material, and to Oliver Warren for preparing some of the slides.

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AN IMPROVED COLORIMETRIC METHOD FOR THE ESTIMATION OF HISTAMINE

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Based upon the diazo reaction given by imidazole compounds, numerous attempts have been made to utilize this procedure for the estimation of histamine. Experience with the method has shown it to be unsatisfactory because of lack of specificity, instability of colors, and because of numerous substances in biological extracts which inhibit or interfere with the reaction (1). We have been able to a large extent to overcome these difficulties and to extend the sensitivity of the test.

After comparing a large number of aromatic amines, the diazonium salt of 4-nitroaniline was selected as the most satisfactory. This compound has been previously employed for this purpose (2).

The colored azo compounds formed by 4-nitrodiazobenzene in alkaline solution are extracted with a small amount of an organic solvent. This serves to concentrate and to stabilize the color. It was also found that at an appropriate pH, and with certain solvents, the azo compounds of most interfering substances would either remain in the aqueous phase or pass into the solvent (methyl isobutyl ketone) with a yellow or amber color, while that of histamine goes into the solvent with a rose color.

A few substances present in biological fluids, notably ammonia, reacted with the diazonium salt to give a compound which, similar to histamine, was extracted into the solvent with a rose color. The color given by ammonia is less stable than with histamine and its pH characteristics are different. By shaking the solvent with a barbitol buffer of pH 7.7, the rose color obtained with histamine or acetyl histamine is intensified while that of ammonia and other interfering substances, so far observed, are abolished or changed to a pale yellow.

Finally, it was necessary to overcome the problem of inhibitory substances. The diazo reaction in alkaline solution is affected by various organic compounds as well as by high concentrations of certain inorganic salts. Confirmatory of other experience (3) uric acid was found to inhibit, in equimolar ratio, the diazo reaction with histamine and with other imidazoles. This inhibition was not exhibited by related compounds, including caffeine, adenine, xanthine, hypoxanthine, theobromine, theophyllin, hydantoin, alloxan, allantoin, guanine, uracil and creatinine.

It was found that brief standing or heating with nitrous acid would overcome the effects of inhibitors present in trichloroacetic acid extracts of most tissues, but not completely for liver extracts or urine. The recently developed method of McIntire, Roth and Shaw (4) for purification of histamine in biological fluids has been found to yield satisfactory results when adapted for the colorimetric estimation of histamine in urine and tissue extracts.

METHODS. Organ extracts were obtained by grinding with sand with a mortar and pestle, while gradually adding 4 volumes of 10 or 20 per cent trichloroacetic acid; with liver 20 per cent trichloroacetic acid was used. The extracts were filtered off after standing 15 minutes. In the case of blood, 7.5 cc. of water were added to 5 cc. of blood, and after a few minutes 12.5 cc. of 20 per cent trichloroacetic acid, dropwise at first, with shaking; a 1 to 5 dilution in 10 per cent trichloroacetic acid is thus obtained.

The diazo reagent is freshly prepared from a stock solution of 0.1 per cent 4-nitroaniline in 0.1 normal HCl kept in a dark glass bottle. 10 cc. of this solution are cooled in a beaker of ice and then 1 cc. of 4 per cent sodium nitrite added.

To 5 cc. of the 10 per cent trichloroacetic filtrates in test tubes, 1 cc. of 4 per cent sodium nitrite is added and the tubes immersed in boiling water for 2 minutes. They are then placed in ice water in a beaker. When cool 1 cc. of the diazo reagent is added and the tube shaken to mix. Alkalinization is carried out as follows: Add 1.25 cc. of 20 per cent sodium carbonate, mix, then add 0.5 cc. of carbonate, mix; wait one minute keeping the tube in ice, and then add 0.3 cc. of 5 normal NaOH and mix. The solution should now have a pH of 10.1 to 10.5. Add 2 cc. of methyl isobutyl ketone, stopper, and shake about 25 times. Replace in ice to allow the ketone to separate off, or if separation is slow, centrifuge the cold solution for less than 1 minute.

With a capillary pipette and rubber bulb, a portion of the ketone is then pipetted into small test tubes containing about 6 cc. of barbital buffer of pH 7.7¹, taking care not to include any of the aqueous phase beneath the solvent. The tubes are stoppered and shaken vigorously. After standing 20 to 30 minutes the color in the ketone is compared with a series of standards.

Standards within the range of 1 to 10 gamma of histamine are prepared in 7 per cent trichloroacetic acid, and run simultaneously with the tissue extracts. Because of the frequent occurrence of yellow colors when the reaction is carried out directly on trichloroacetic filtrates of tissues, estimations have been made by direct comparison, with a series of standards, rather than by colorimetry. Daylight is preferable, although artificial light can be employed with appropriate filters.

Stability of colors: The color in the ketone is sufficiently stable; the loss of intensity was measured in a photoelectric colorimeter using a 510 filter, and it was found to be about 7 per cent in one hour, and about 25 per cent in 24 hours.

When the concentration of histamine is varied, estimations with the photoelectric colorimeter indicate that the color intensity follows Beer's law.

Sensitivity of the reaction: As outlined, the procedure can detect 0.5 to 1 gamma of histamine base in pure solution. By reducing the amount of ketone employed to 0.5 to 1 cc., correspondingly smaller amounts can be detected; this procedure also concentrates interfering yellow colors, so that it is feasible only where purification adequate to eliminate these colors is possible.

Attempts to apply the test to volumes of solution larger than 5 cc. have not been satisfactory.

Purification of tissue extracts and urine: In urine, and in the trichloroacetic filtrates of certain organs, notably liver, preliminary purification is required because of the presence of interfering yellow colors and of inhibitors to the color reaction.

Lead precipitation: Where a high degree of accuracy is not required, some degree of success has been attained by lead precipitation, modified from the procedure of Loeper, Lesure, and Thomas (5). Our method is as follows:

To 1 to 5 cc. of urine made up to 5 cc. in water add 1 cc. of a saturated aqueous solution of lead acetate, cool in ice, and add dropwise with shaking sufficient 5 normal NaOH to attain a pH of approximately 8.8, as indicated by thymol blue or the glass electrode. This requires approximately 0.35 cc. in most instances. The solution is centrifuged for a few

¹ The M/20 barbital buffer is prepared by dissolving 10 grams of sodium barbital in 975 cc. of water and adjusted to pH 7.7 with molar (6%) acetic acid (approximately 25 cc.).

minutes and the supernatant fluid decanted into a small graduate. To 5 cc. add 2.5 cc. of a saturated solution of sodium borate and centrifuge. To 4.75 cc. of the clear supernatant fluid add 0.25 cc. of 10 normal H_2SO_4 , cool, and centrifuge if needed. 2.5 cc. of this solution represents 1.25 cc. of the original. Because of the high concentration of inorganic salts the intensity of the color reaction may be inhibited and it is preferable to dilute this solution with equal parts of 0.4 N HCl or H_2SO_4 before applying the diazo reaction.

In the case of liver or other tissue extracts in 20 per cent trichloroacetic the same procedure is used for 5 cc. aliquots, except that 0.5 cc. of saturated lead acetate is used, approximately 1.0 cc. of 5 N NaOH is required, and only 1 cc. of saturated sodium borate is needed. 5 cc. of the final solution represents 3 cc. of the original extract. The standards are made up in 14 per cent trichloroacetic and put through the same treatment.

This procedure has been found useful under experimental conditions where high concentrations of histamine in the tissues or urine permit considerable dilution of the final solution. For urines with much ammonia or little histamine it is not satisfactory.

Cotton acid succinate method: Preliminary experience with the cotton acid succinate (CAS) method indicates that it may be applied to urine and to tissue extracts with satisfactory results for the colorimetric estimation of histamine. The solution to be tested is treated with a salt mixture of Na_2SO_4 and Na_2PO_4 and extracted with butanol. At room temperatures below 25°C. we have frequently encountered insolubility of the salt mixture; slight warming is necessary to bring the salts into solution. The histamine is adsorbed from the butanol upon CAS, which is then washed with alcohol and water. The histamine is then eluted from the CAS by 0.4 normal HCl. Details of this procedure may be found in the publication of McIntire, Roth, and Shaw (4).

In the preparation of the CAS pads it is necessary to wash them free from acid with considerable quantities of water. The distilled water which was available to us contained traces of substances that were inhibitory to the color reaction. These substances were concentrated on the pads and eluted by acid along with the histamine so that at first it was not possible to obtain complete recovery of added histamine.

Analysis indicated that the chief inhibitory factor was copper, which will interfere if present in amounts equivalent to the histamine. This observation also serves to illustrate the necessity of employing clean glassware and reagent grade of chemicals throughout the procedure. Some filter papers have been shown to contain traces of heavy metals.

The difficulty with water was obviated by careful redistillation in pyrex glass, or by passing the distilled water through a 6 inch column of the cotton acid succinate containing about 1 gram of this material. After each 10 to 20 liters the column should be purified by washing with 0.4 N HCl, followed by sufficient water to remove the acid.

As applied to tissue extracts and urine, certain modifications are necessary. It has been found that histamine is eluted from the CAS by inorganic salts; the presence of ammonia in excess of 1 mg., or of considerable amounts of NaCl, or greater than 3 mg. of trichloroacetic acid in the original solution will render the method unworkable. Sodium sulfate in the original solution does not interfere. Before employing trichloroacetic acid extracts it is therefore necessary to remove the acid.

To accomplish this add 0.1 cc. of 5N H_2SO_4 to 10 cc. tissue extract; repeatedly (4-6 times) extract the solution with 30 cc. reagent grade ether (minimal peroxide content) in separatory funnels. Satisfactory removal of the trichloroacetic acid is indicated if an aqueous solution of brom cresol green is not decolorized when shaken with the final ether layer. Traces of ether are removed from the aqueous layer by aeration; this is then approximately neutralized with 0.1 cc. of 5N NaOH.

For the determination of free histamine in the urine, 1 to 4.5 cc. (made up to 4.5 cc.) are used, depending on the concentration of urine. After the addition of the salt mixture and butanol, most of the ammonia should be removed by aeration for $\frac{1}{2}$ hour.

A further modification is required in the detection of conjugated histamine, to be discussed below. Conjugated histamine is partially adsorbed from butanol upon the CAS, but it is more easily eluted than free histamine so that a variable amount is removed when

the CAS pad is washed with alcohol and water. It has been found that after washing the pad with alcohol, washing with 1 cc. of 0.014 M Na_3PO_4 , followed by the usual 3 cc. of water, will completely elute the conjugated histamine (in amounts less than 100 γ) without removing the free histamine. The nature of the conjugate has not been established, but it is of interest that N-acetyl histamine behaves similarly to the urinary compound.²

The colorimetric method will not differentiate free from conjugated histamine. By applying the above modification to the CAS method the final HCl eluate can be employed for the estimation of free histamine by the colorimetric method.

In order to determine the conjugated histamine in the urine by means of the CAS pads, it is necessary to convert it to free histamine by acid hydrolysis. This treatment liberates ammonia from urea, and since considerable amounts of ammonia interfere with the absorption of histamine by the CAS pad, it is necessary to remove most of the ammonia by aeration. The following procedure has been employed:

To 10 cc. of urine are added 2 cc. of 10 N sulfuric acid; this is hydrolyzed in boiling water or a steam bath for 3 hours, with the addition of water if evaporation approaches dryness. It is then cooled, made up to 5 cc. and centrifuged. 2.5 cc. of the supernatant fluid are placed in a large test tube, and approximately 0.4 gm. of $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ added. After it are dissolved, the tube is placed in ice water and 2.1 cc. of 5 N NaOH added drop by drop with shaking. 5 cc. of butanol are now added and a vigorous stream of nitrogen (hubbled through water) passed through the solution for 1 hour. A conventional glass bulb with small holes has been used for this purpose. At the end of 1 hour the aeration bulb is held at the mouth of the test tube and washed with 0.5 cc. of butanol. Approximately 300 mg. of powdered anhydrous Na_2SO_4 is now added, the tube tightly stoppered with a glass or cork stopper, and shaken vigorously in a shaking machine for one half hour. The tube is briefly centrifuged and a measured amount of the butanol, 4.5 to 5 cc., is put through the CAS pad as described by McIntire, Roth, and Shaw (4). The remaining butanol must be measured, to calculate the percentage represented.

The value after hydrolysis represents total histamine; the difference between this and the value for free histamine, determined before hydrolysis, represents conjugated histamine.

The diazo reaction can be applied directly to the final eluate from the CAS pad in 0.4 N HCl. 2 cc. of acid can be used followed by 0.5 cc. of water. For 2.5 cc. containing 2 cc. of HCl the quantity of Na_2CO_3 required in the alkalization should be reduced to 0.3 cc. plus 0.2 cc., followed by 0.12 cc. of 5 N NaOH; otherwise the procedure is similar to that described above, using half the amounts of reagents described for an initial volume of 5 cc. Following CAS purification the color match is usually satisfactory for the use of photoelectric colorimetry.

It is also possible to utilize histaminase to differentiate between free and conjugated histamine. Anrep has demonstrated that conjugated histamine is not attacked by the enzyme (6); by permitting histaminase to act upon diluted urine samples or upon tissue extracts from which the acid has been removed, the colorimetric procedures can be applied to determine the amount of free histamine destroyed.

SPECIFICITY OF THE METHOD. Tests have been carried out on various metabolites in concentrations that may be encountered in the body. The following compounds yield a red, orange or yellow color in the aqueous phase, but a yellow color or none at all in the ketone: Histidine, acetyl histidine, carnosine, imidazole, pyridoxin, phenol, 4-hydroxy phenyl acetic acid, 4-hydroxy phenyl glycine, epinephrine, dihydroxyphenyl alanine, tyrosine, tyramine, acetyl tyramine, p-cresol, tryptophane, indol, guanine, uric acid, peptone (Witte), hematin, bilirubin.

*The urinary conjugate from several species of laboratory mammals has behaved in this manner; studies have not yet been carried out in man.

Other metabolites tested give no color in aqueous phase or solvent. Ammonia gives a yellow color in the aqueous phase but a rose color in the ketone. As outlined in the method, this color is dissipated by washing the ketone with barbitol buffer. Large amounts of ammonia, 2 to 5 mg. require a half hour for the disappearance of the color.

TABLE 1

Colorimetric determination of histamine in trichloroacetic acid filtrates of tissue

The tests were performed directly on the filtrates except in the case of liver and some kidney filtrates, where further precipitation with lead acetate was used. Figures in the third column represent individual determinations. Values below 1 gamma signified as trace (T) or negative. Values expressed as histamine base.

ANIMAL	ORGAN	HISTAMINE <i>gamma/gm.</i>	AVERAGE
Guinea pig	Lung	20, 16, 14, 13, 7, 16	14.3
	Blood	0, 0, 0, 0, 0, T	0-T
	Spleen	2.4, 2.0, 1.6	2.0
	Kidney	0, 0, T, T	0-T
	Testis	0, 0, 0	0
	Muscle	0, T, 1	0-1
	Liver	0, 0, 0	0
Rabbit	Lung	3, 5, 1.5	3.2
	Blood	1.25, 1.5, 1.5, 3, 1.3, 2.8, 3.4, 3.4, 3.3, 4, 2.5, 3.5	2.55
	Plasma	T, T	
	Liver	0	
	Kidney	2	
	Muscle	0.5, 0	
Rat*	Lung	5, 5	
	Blood	0, T	
	Liver	0, 0	
Mouse†	Lung	0, 0, 0, T, T, 1	0-1
	Blood	0, 0, T, T	0-T
	Liver	0, 0	
Dog	Liver	25, 30	
	Muscle	3, 7	
	Lung	43, 25	

* Pooled organs of 2 rats for each determination

† Pooled organs of 10 mice for each determination

N-Acetyl histamine, 4 [β -acetyl aminoethyl] imidazole, behaves similarly to histamine and gives a rose color comparable in intensity and shade.

Further proof of the specificity of the method was afforded by histaminase. Two preparations of a partially purified enzyme were found to destroy the imidazol ring in histamine, as indicated by the disappearance of the diazo reaction

the CAS pad is washed with alcohol and water. It has been found that after washing the pad with alcohol, washing with 1 cc. of 0.014 M Na_2PO_4 , followed by the usual 3 cc. of water, will completely elute the conjugated histamine (in amounts less than 100 γ) without removing the free histamine. The nature of the conjugate has not been established, but it is of interest that N-acetyl histamine behaves similarly to the urinary compound.²

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*The urinary conjugate from several species of laboratory mammals has behaved in this manner; studies have not yet been carried out in man.

Rather than a systematic survey of histamine content, these determinations represent a trial of the procedure with various tissues. Results with liver (except dog liver) and kidney are only approximate because of low content and poor color match. The values obtained are in good agreement with recent reports of histamine content of some of these tissues, determined by biological assay (1, 7).

In a few experiments upon guinea pig lung and dog liver no conjugated histamine could be demonstrated by the use of histaminase.

FREE AND CONJUGATED HISTAMINE. Anrep and co-workers made the important observation that orally administered histamine in dogs is excreted in appreciable quantity in the urine in a conjugated form (6).

We have confirmed this finding in dogs, guinea pigs, rats and rabbits. In our experiments histamine given to dogs by stomach tube produced frequent vomiting and diarrhea, which interfered with quantitative determination. In table 2 are reported the results of two experiments where preliminary administration of morphine (10 mg. per kilo, s.c.) was used to prevent vomiting. Three hundred and thirty mg. of neutralized histamine dihydrochloride (200 mg. of the base) were given in 250 cc. of fluid. Nine and 20 per cent were recovered in the urine, almost entirely as a conjugated compound, as shown by its behavior towards histaminase and towards cotton acid succinate. After acid hydrolysis it behaved as free histamine.

SUMMARY

A colorimetric method for the detection of histamine has been developed. It has been adapted for the determination of free and conjugated histamine in animal tissues and body fluids.

Preliminary results upon various tissues, and with histaminase, indicate a satisfactory degree of specificity.

The sensitivity of the method is approximately 0.5 microgram.

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in both the aqueous phase and solvent. The specificity of the enzyme was indicated in that acetyl histamine, imidazole lactic acid, carnosine, imidazole, and histidine were not attacked.

Extracts of lung and liver giving the color reaction were freed from trichloroacetic acid; when subjected to histaminase the reaction was abolished. Substances in urine which behave toward cotton acid succinate as conjugated histamine were not affected by histaminase, as indicated by the diazo reaction; after acid hydrolysis of the urine, histaminase abolished the color reaction. Likewise, histaminase abolished the color reaction of acetyl histamine only after acid hydrolysis.

The separation of the azo derivatives of histamine from histidine and related compounds with carboxy groups depends on the insolubility of the alkali salts

TABLE 2

Recovery of histamine in urine of a dog after 200 mg. by stomach tube

Colorimetric determinations made following purification by lead acetate precipitation or by the cotton acid succinate method. The histamine behaved as a conjugate.

EXPERIMENT NO.	HOURS AFTER HISTAMINE	URINE VOLUME	HISTAMINE	HISTAMINE TOTAL
		cc.	gamma/cc.	mg.
1	0	40	0	
	6½	50	20	1.0
	24	75	300	22.5
	32	42	300	12.6
	48	180	20	3.6
				39.7
2	6½	45	40	1.8
	24	190	70	13.3
	32	110	12.5	1.37
	48	86	12.5	1.07
				17.54

of the latter compounds in the ketone at the pH employed. It is likely that certain substitutions of these carboxy groups, as by esterification, may interfere with this separation. Likewise, certain closely related derivatives of histamine such as 4[β -hydroxy ethyl] imidazole, may also give compounds soluble in the ketone. However, the evidence so far obtained indicates that they are not present in the body in amounts detectable by this procedure.

RESULTS IN ANIMAL TISSUES. Table 1 contains some preliminary results upon trichloroacetic acid extracts of organs of several species, expressed in micrograms per gram of fresh tissue. Negative values mean that the concentrations were too low to be detected by the method, less than 0.5 to 1 gamma. Where little or no histamine was found the validity of the results was checked by the recovery of 1 to 2.5 gamma of histamine added to the filtrate or to the original tissue.

Rather than a systematic survey of histamine content, these determinations represent a trial of the procedure with various tissues. Results with liver (except dog liver) and kidney are only approximate because of low content and poor color match. The values obtained are in good agreement with recent reports of histamine content of some of these tissues, determined by biological assay (1, 7).

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THE RESPONSE OF ISOLATED HYPODYNAMIC MYOCARDIUM TO INOTROPIC DRUGS¹

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To A. J. Clark (1) is largely due modern interest in the response of cells to drugs and the realization that the basic problems in drug action must be studied at this level. In previous communications from this laboratory (2, 3), it was pointed out that many of the quantitative evaluations of inotropic activity reported for the so-called cardiac glycosides are based upon toxic or lethal effects in the intact animal. As Gold (4) has pointed out, it might be better to measure a beneficial or therapeutic effect rather than toxicity. It is interesting, also, to know the potency of pure cardiotropic substances in terms of their direct effect upon isolated myocardium.

For these reasons the restored response of hypodynamic muscle has been applied in a semi-quantitative procedure, which might be analyzed statistically. To this end the test tissue selected was the isolated papillary muscle of the right ventricle of the cat heart, as described originally by Cattell and Gold (5). It was pointed out in a previous communication (2) that this technique could yield approximate values for equivalent quantities of pure inotropic substances, when assayed in extremely small amounts. It is the purpose of the present report to discuss certain characteristics of this myocardial preparation as used to test relative inotropic effects.

EXPERIMENTAL PLAN. In comparing an "unknown" glycoside with crystalline U.S.P. reference standard ouabain (11% water), it is necessary to design the experiment *foresightedly* so that the unknown solution will match the standard closely. In computing the optimal concentration to be used of the unknown solution, two features are considered. The first feature is that, to match one unit of standard, E units of unknown will be required. The second feature is that each successive dosage of glycoside should increase by a regular interval which is $\log I = i$. For example, in matching 5 micrograms per cent of ouabain, $5 \times E \times I$ micrograms per cent of the unknown would be used. In actual fact ordinarily these two factors (which were known as the E and i factors, respectively) remained "hidden" until the assay was over. Therefore, once the known and unknown solutions were made up, they could be tested in pairs *seriatim* at the same titular value. In practice, if the Equivalence factor is well known, the unknown solution should be slightly more potent

¹ For the pure substances used in this study the authors are especially indebted to Dr. K. K. Chen of the Eli Lilly Company, who contributed the major series and offered helpful suggestions. They are also indebted to Harry Gold, Dr. A. E. Farah and to Dr. S. M. Fossel of Sandoz Chemical Works, Inc. for certain individual samples.

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than the standard, because of the i factor: therefore it would give a somewhat greater response. This maneuver will be readily understood from a typical protocol, shown in table I. Although some randomization of dosage was possible by alternating the order in which known and unknown were tested, it appeared necessary to avoid a recession in potency in any couplet of successive doses.

METHODS. The experimental procedure has been described in detail in previous communications (2, 6). The mammalian Ringer's medium employed contained 0.1% glucose but no added carbonate. The chief buffer used was 0.154 M phosphate at pH 7.2. The pure cardiotropic substances were made up in 1 to 1000 hydro-alcoholic solution, preserved in sterile ampules, each capped with a soft rubber diaphragm through which a hypodermic needle could be inserted. The hypodermic needle was affixed to a calibrated syringe-pipette. The solvent used was 47.5% ethyl alcohol (by volume). The ampules were stored

TABLE I

Comparative inotropic responses of thevetin and hydrated ouabain, respectively

Muscle #33.

Assumed E factor = 6.67 (Reciprocal of assumed relative potency).

Selected I factor = 1.123; $\log I = i = 0.050$.*

Hydrated ouabain (11% water) as standard.

TITULAR OR PRETENDED CONCENTRATION	ACTUAL CONCENTRATION	ORDER OF TESTING	PHOTOGRAPHED CONTACT- TILE RESPONSE	RESPONSE AS PER CENT OF MAXIMAL
$\mu\text{g./100 cc.}$	$\mu\text{g./100 cc.}$		$\text{mm.} \times 100$	
Ouabain				
0	0		37.4	—
2.51	2.51	(1)	37.4	—
3.16	3.16	(2)	37.4	63.8
3.98	3.98	(4)	48.8	83.3
5.01	5.01	(6)	58.5	100
Thevetin				
3.16*	23.7	(3)	45.5	77.7
3.98	29.9	(5)	58.0	99.1
5.01	37.6	(7)	toxic	toxic

$M' = 2.0329$.

Antilog $M' = 107.9\%$ for equivalence value $E = 6.67$.

True $E = 6.18$.

* Note that for the first concentration of Thevetin, $E \times I = 23.7/3.16 = 6.67 \times 1.123$.

in an icebox at 4°C. Before sampling, however, they were warmed up to 20°C. In the use of the calibrated syringe-pipette, it was essential to express the contents with a single, steady stroke of the piston. Under these circumstances, as was proved by direct trial, an extremely accurate delivery of the solution could be made.

Ordinarily the test solution, as made up just prior to the bioassay, consisted in 0.281 cc. of stock solution diluted in a small volumetric flask to the appropriate dilution. This dilution varied with the substance involved but ordinarily the total volume was about 25 cc. The diluent was glass-distilled water. Every effort was made to treat all the drugs in the series alike, and especially in any one experiment to handle the standard ouabain and the unknown in precisely the same manner. In addition to the arrangement of the apparatus previously described (2) it was found possible to study simultaneously two papillary muscles from the same animal. These muscles were housed in separate chambers, but their respective contractions were flashed onto the same camera. The solution around the

muscle was replaced by a fresh one containing the drug at each dose level. A characteristic experiment is illustrated in fig. 1.

THE HYPODYNAMIC STATE. Fresh papillary muscles, vigorously contracting, will not respond to cardiac glycosides, except by toxic rigor when high dosage is resorted to. In other words, it is essential that the myocardium be definitely abnormal before it will respond to "therapeutic" concentrations. It is surprising how many biologists have criticized this preparation because the tissue was not fresh. The necessity of abnormality, however, should not be surprising to those who realize that the digitalis glycosides act characteristically only in myocardial failure. Eventually, after many hours, these muscles show irreversible deterioration as indicated by a steady decline in the amplitude of contraction under stable conditions. In some instances the decline is more rapid than in others

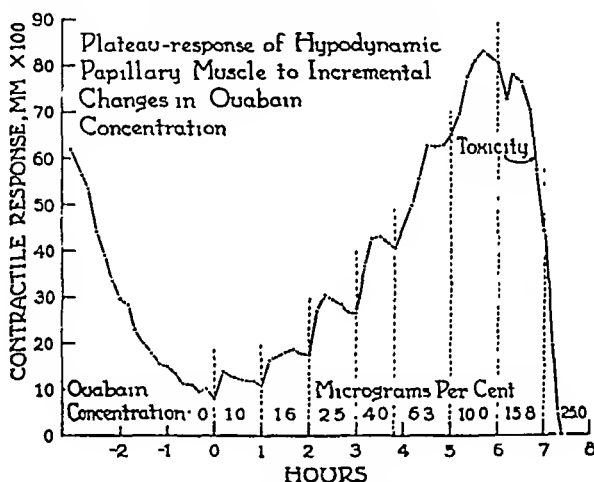


FIG. 1. AFTER EXPOSURE TO AN INCREMENTAL INCREASE IN OUABAIN CONCENTRATION, THE RESPONSE OF THE PAPILLARY MUSCLE REACHES A NEW LEVEL WITHIN 20 TO 90 MINUTES, DEPENDING UPON THE GLYCOSIDE UNDER STUDY

In judging a contractile response, therefore, some information is needed as to the general trend of the muscle's contractility. This point is illustrated by comparison of figs. 1 and 2. Rarely, a muscle will be encountered which shows a gradually increasing contractility over a period of several hours.

In the communication which immediately follows this report (7), glycosides of unknown potency were alternated with ouabain. Accordingly, provided that deterioration was progressive, the responses both to the known and the unknown would be influenced approximately alike. In actual fact, the maximal contractile response observed in many instances *exceeded* that seen originally in the fresh muscle, even though ten or more hours had elapsed.

THE TIME FACTOR. In routine assays upon intact animals, as Maresh and Farah (8) have pointed out, an arbitrary time interval has been adopted in most

cases. Consequently no steady or equilibrium condition has resulted throughout such an experiment. Farah met this difficulty in intact cats by decreasing the rate of administering cardiotoxic drugs until the result was independent of time. In the case of the papillary muscle, too, the influence of time has been minimized by observing the contractile response after each increase in dosage at 10- to 15-minute intervals until the amplitude became constant. Ordinarily this required 60 to 90 minutes, as shown in fig. 1. Investigators working in this field may wonder at such speedy equilibration. It must be remembered, however, that the increase in inotropic response as elicited in these experiments proceeded by only very short increments: so that relatively little adjustment in contractility occurred at each stage.

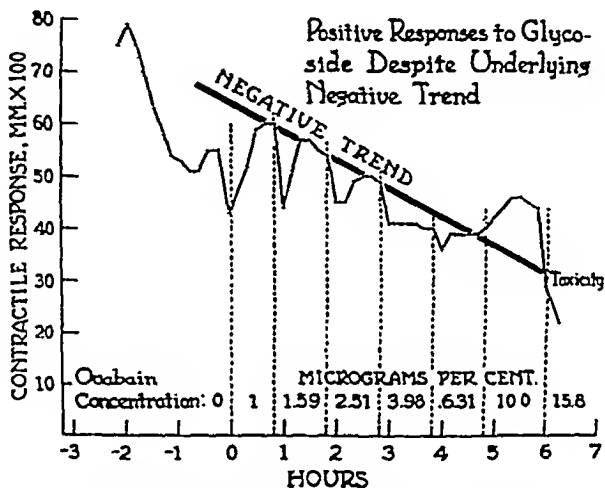


FIG. 2. PROBABLY DUE TO EXCESSIVE TENSION ON THE PAPILLARY MUSCLE, THIS UNUSUAL PREPARATION SHOWED PROGRESSIVE DETERIORATION

Nevertheless, the incremental changes in glycoside concentration each produced a response. Obviously preparations of this sort would not be used in an assay.

The effect of time was easily apparent with an incremental dosage which just exceeded the optimal. In such instances the maximal contraction occurred in approximately 45 minutes and after 90 minutes had already declined perceptibly,—even below the initial response at that dosage.

REVERSIBILITY. It has been stated frequently that certain glycosides are fixed irrevocably within the muscle, once they have produced their effect (9). Nevertheless, under the conditions of the present experiments a decline in contractility has been found regularly when the solution of glycoside was replaced by pure Locke's medium. Moreover, in comparing two glycosides, occasionally too low a concentration of the second has replaced the first. Such a negative response is illustrated in fig. 3 for cerebroside. In such a case the amplitude has declined by a reasonable proportion. The phenomenon was observed

both with ouabain and digitoxin, and with several other glycosides. It must be emphasized, however, that such a recessive contractility involved only a minor change in the effective concentration of drug. Usually such situations were avoided.

INTERCHANGEABILITY OF GLYCOSIDES. No evidence of potentiation could be demonstrated in these studies. For example, when a certain concentration of ouabain was replaced by an isodynamically equivalent concentration of digitoxin, the contractile response remained steady, as shown in fig. 4. When mixtures of glycosides were studied, the net effect could be predicted as a summation of the constituents of the mixture, calculated in terms of isodynamic equivalence. For example, if approximately 2.5 micrograms of digitoxin are required to replace 1 microgram of (hydrated 11%) ouabain, then a mixture of

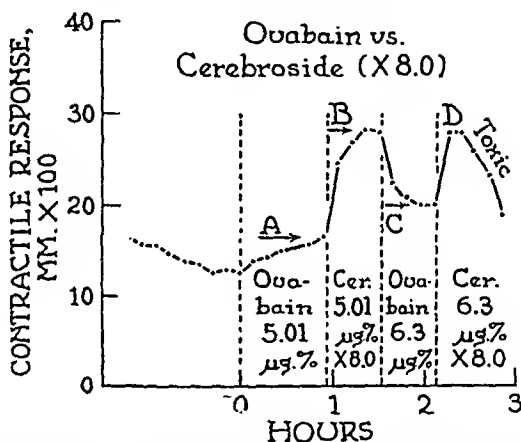


FIG. 3. THE GRAPH DEMONSTRATES TWO PHENOMENA: (1) THE DECREASED RESPONSE, C TO A LOW CONCENTRATION OF OUABAIN AFTER A HIGHER RESPONSE, B, TO A LARGE CONCENTRATION OF CEREBROSIDE; (2) THE FINAL TRANSITION ON THROUGH A MAXIMAL LEVEL, D, BEFORE TOXICITY OCCURS FROM A SINGLE DOSAGE

1.25 micrograms of digitoxin plus 0.5 microgram of ouabain should be equivalent to 1 microgram of ouabain. By actual test, the potency was 106% that expected—and not half. Indeed, the isolated papillary muscle offers a good opportunity to study summation.

COMPARISON OF INDIVIDUAL MUSCLES. When the contractile responses of several individual muscles are plotted against dosage, the first plot is bewildering. Fig. 5, for example, shows data for four papillary muscles treated alternately with convallotoxin and ouabain. The results, however, can be homogenized by adopting two maneuvers:

(1) The contractile responses should be expressed as *fractional* responses (or percentages) of the maximal. This adjustment gives every plot a common "ceiling," so that the curves all approach a common slope. Even so, the ouabain

responses for individual muscle are still displaced from one another by various intervals along the abscissa. So, too, are the homologous curves for convallotoxin. Therefore,

(2) Each ouabain curve should be set arbitrarily so that a certain response corresponds to a log-dose of 1.0. Then the convallotoxin curves will tend to fall superimposed, because each differs from its respective homologous standard curve by the same potency-interval.

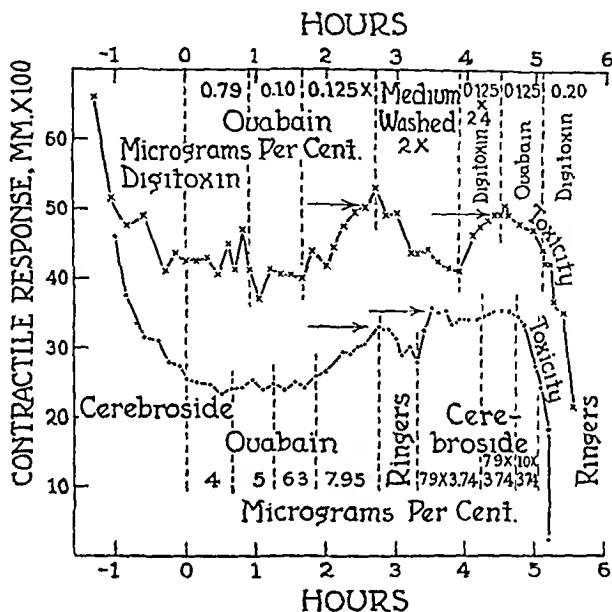


FIG. 4. WHEN ISODYNAMIC CONCENTRATIONS OF CARDIAC GLYCOSIDES ARE SUBSTITUTED FOR A PREVIOUS SOLUTION, THE PAPILLARY MUSCLE EXHIBITS APPROXIMATELY THE SAME RESPONSE; ALTHOUGH THERE MAY BE A SLIGHT TREND UPWARD OR DOWNWARD AS TIME PASSES

Note the decline in response when Locke's solution (without any drug) is substituted.

The results compiled by these maneuvers indicate that in the series of congeners studied the common slope is 1.0, approximately. In other words, in respect to the preparation under study a change in log-concentration of one unit carries the hypodynamic muscle from minimal to maximal contractility. This observation was tested with 9 papillary muscles, for each of which the relative potency of digitoxin was calculated by two methods, i.e.,

- by classical two-by-two statistical procedures (11): and
- by the maneuvers described above.

Representative data are given in table II. For example, observe Muscle 6E. For ouabain, the average log-dose was 0.55 and the average fractional response 0.562. Therefore, this muscle was "misplaced" by a log-dose interval of ± 0.012 .

Referring to the digitoxin data on the same muscle, the average fractional response was 0.625 for an average log-dose of 1.10. By difference the log-potency was 0.375. This last value, however, must be corrected for "misplacement" by the muscle constant of $+0.012$. Hence the corrected log-potency is 0.387 and the Equivalence values is its antilog, i.e., 2.43. The classical statistics give the value 2.46. Thus the group of nine muscles can be compared as shown in table III.

These computations indicate that ouabain may be used to calibrate the individual papillary muscle; and from each muscle constant so derived the potency of an unknown glycoside can be estimated. As yet there is no clue as to the meaning of the "misplacement" indicated by the individual muscle constant.

RESPONSES OF FOUR INDIVIDUAL PAPILLARY MUSCLES

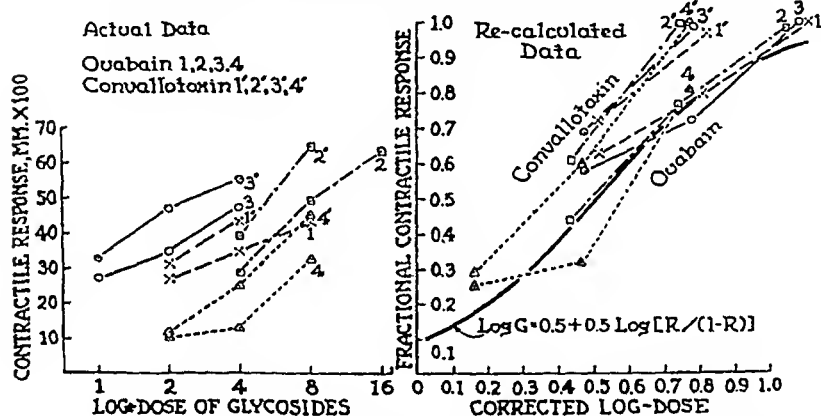


FIG. 5. THE RESPONSES OF FOUR PAPILLARY MUSCLES TO CONVALLOTOXIN AND OUABAIN PRESENT A CONFUSED PICTURE UNTIL THE DATA ARE TRANSPOSED BY CALIBRATING EACH PREPARATION BY MEANS OF THE OUABAIN DATA

In the equation represented by the heavy line, G signifies the concentration of ouabain and R the fractional response (referred to the maximal).

Note that in the left-hand figure the scale is logarithmic, but has been labeled with the corresponding arithmetical equivalents in micrograms percent.

GENERAL EQUATION. This concept can be extended to a series of closely related cardiac glycosides to formulate a general equation (3) relating fractional contractile response to concentration of drug. The ideal glycoside G evokes a maximal inotropic effect when log-concentration equals unity = $\log 10$. Therefore the "therapeutic" effect follows the equation:

$$\log G = 0.5 + 0.5 \log [R / (1 - R)]$$

where R signifies any given contractile response expressed as a fraction of the maximal. In a practical instance, $\log G$ becomes $(\log G + \log M + \log a)$, where $M = -\log$ potency, and a is the constant characteristic of the individual sample of myocardium under observation. It is convenient to assign to ouabain

MUSCLE	OUABAIN			DIGITONIN			RECIPROCAL OF POTENCY
	Log dose	Response as photographed	Fractional response	Log dose	Response as photographed	Fractional response	
6 E	0.00	mm. 34.0	0.330	0.6	mm. 37.3	0.362	
	0.4	38.5	.374	1.0	46.4	.451	
	0.8	60.0	.533	1.3	71.0	.688	
	1.0	99.0	.962	1.5	103.0	1.000	
Average.....	0.55		0.562	1.1		0.625	2.46
15 E	0.6	38.4	0.802 .946	1.05 1.35	41.0 47.8	0.856 1.000	
	0.9	45.3					
Average.....	0.75		0.874	1.20		0.928	2.45
18 E	0.0	20.5	0.297	0.46	20.8	0.301	
	0.3	23.0	.333	0.76	25.0	.362	
	0.6	32.0	.464	1.06	40.3	.584	
	0.9	56.0	.811	1.36	66.4	.961	
	1.1	69.0	1.000				
Average....	0.59		0.581	0.908		0.552	2.21
2-7	0.7	18.0	0.546	1.146	31.0	0.712	
	0.9	23.5	.938	1.342	33.0	1.000	
Average... .	0.80		0.743	1.244		0.856	2.40
1-G	0.3	37.3	0.541	0.9	48.6	0.705	
	0.7	58.0	.842	1.3	69.0	1.000	
Average . .	0.50		0.692	1.097		0.853	2.39
4-G	0.5	6.3	0.443	0.7	5.7	0.401	
	0.9	14.2	1.000	1.1	9.6	.676	
Average.....	0.7		0.722	0.898		0.539	2.38
3-G	0.5	62.3	0.763	0.7	56.8	0.605	
	0.9	81.7	1.000	1.1	69.4	.849	
Average	0.7		0.882	0.898		0.772	2.36
5-G	0.5	26.4	0.931	0.8	18.1	0.637	
	.699	28.0	.986	1.0	28.4	1.000	
Average	0.6		0.956	0.898		0.818	2.57
7-G	0.6	44.0	0.641	0.898	41.0	0.596	
	0.8	68.7	1.000	1.097	57.7	0.839	
Average	0.7		0.821	0.997		0.718	2.71
Average.....							2.44 ± 0.14

(11% water) the arbitrary value $M = 0.285$. Then its E.D.₈₀ reads 1.0. Technically it seems sounder to determine the E.D.₈₀ than the E.D.₅₀, because usually the experimental data are clustered about this level.

INFLUENCE OF THE MEDIUM. As pointed out by Sollman, von Oettingen and Ishikawa (12) the ionic constitution of the medium is important in studies such as these. Of special interest are the calcium, phosphate and hydrogen ions. Artificially buffered solutions tend to precipitate calcium until a pH value of 7.2 is assumed spontaneously. In order to avoid precipitating calcium, it is convenient to maintain the pH at 7.2 and to avoid phosphate concentrations higher than 0.154M. Lest some critics might be skeptical of pH 7.2, however, certain experiments were conducted specially at 7.4 as tested by a glass electrode. The following protocol indicates that the inotropic response occurs equally well and indifferently at pH 7.0, 7.2 or 7.4, respectively.

TABLE III
Equivalence of digitoxin (per unit hydrated ouabain)

MUSCLE	CLASSICAL COMPUTATION	FROM INDIVIDUAL MUSCLE CONSTANT
6 E	2.46	2.43
15 E	2.45	2.49
18 E	2.21	2.28
2-7	2.40	2.14
1 G	2.39	2.73
4 G	2.38	2.40
3 G	2.36	2.03
5 G	2.57	2.75
7 G	2.71	2.51
Average	2.44	2.41

When carbonate is employed instead of phosphate, the medium tends to become alkaline during oxygenation and the muscle contracts maximally for long hours, thus remaining useless for studies of the inotropic response. The same phenomenon occurs even in the presence of 5% carbon dioxide. This finding suggests that the presence of carbon dioxide specifically protects the muscle against deterioration, quite apart from its influence on the pH value. Of course the presence of oxygen is highly essential for muscle contractility, and without adequate oxygen the inotropic effect of the cardiac glycosides fails to appear.

QUALITATIVE DISTINCTIONS. For the investigator interested in the effect of drugs directly upon the myocardium, the results obtained in the intact animal have no immediate application. Accordingly, one must be cautious in translating results from one type of experimentation to another. For example, digilanid C is highly potent in the intact animal; whereas on the papillary muscle it exerts but an indifferent effect. The indication, of course, is that the digilanid must be hydrolyzed somewhere in the intact organism before it can affect the hypodynamic myocardium. One must be wary also of concluding

that the special kind of hypodynamic state produced in these experiments reflects clinical heart failure directly. Only careful experimental comparisons will justify such a conclusion. For the moment one must be content to see that closely related congeners of digitoxin restore the response of hypodynamic myocardium in similar fashions, which can be predicted approximately in terms of a general equation.

TABLE IV
Lack of influence of minor variations in acidity

EXPT. NO.	pH	GLYCOSIDE LOG-CONC. (EQUIVALENT)		EQUIVALENCE		
		ouabain	cereberoside	Contractile response	Assumed	Found
27G Neutral	7.51-7.34	—	4	12.8		
	7.50-7.32	5.0	—	14.1		
	7.50-7.30	—	6.3	20.2		
	7.50-7.22	7.9	—	25.4		
					3.9	3.6
23G Acid	7.00-7.01	—	3.16	37.5		
	7.00-6.99	4.0	—	38.9		
	7.00-6.99	—	5	44.5		
	7.00-6.95	6.3	—	47.9		
	7.00-6.97	—	10	57.8		
	7.00-6.96	12.5	—	66.0		
					3.9	3.6

SUMMARY

The inotropic response of the isolated papillary muscle of the cat's right ventricle has been studied in relation to increasing concentrations of "cardiac glycoside." In a series of congeners of digitoxin it has been possible to obtain characteristic and reproducible results under certain specified conditions. Among the essential conditions is the preliminary production of a hypodynamic state; fresh muscle will not respond to digitoxin congeners in the "therapeutic" range of concentration. After calibration with a ouabain standard each individual papillary muscle can be assigned its own muscle constant, a . Then the fractional response, R , to any glycoside G , follows the equation:

$$\log G + \log M + \log a = 0.5 \log < R/(1 - R) >$$

where $\log M$ is the negative log-potency of the glycoside.

The authors wish to acknowledge helpful advice from Dr. McKeen Cattell.

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ISODYNAMIC EQUIVALENTS OF DIGITOXIN CONGENERS AS TESTED ON HYPODYNAMIC MYOCARDIUM¹

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In the communication immediately preceding this (1) were reported the characteristics of the isolated papillary preparation of Cattell and Gold (2) as adapted to quantitative studies. It is the purpose of the present communication to report the values so obtained for a number of pure compounds and to contrast these values with well authenticated values already in the literature based upon the lethal effect in intact cats. As will appear, the relative potency of digitoxin congeners tested on isolated myocardium does not always agree with analogous values determined in the intact animal. Nevertheless, the over-all picture yielded by either method is approximately the same.

EXPERIMENTAL PLAN. The cardiotropic substances under test were tried against the crystalline U.S.P. reference standard ouabain which contains approximately 11% of water. The muscle was stimulated electrically with a spike-exponential-wave thyrotron device delivering from 2 to 60 volts at approximately a frequency of 60 per minute. After a preliminary period of electrical stimulation during which the response of the papillary muscle declined markedly, the perfusing mammalian Ringer's medium (3, 4) was replaced with an isotonic solution containing a known concentration of the substance under test. Alternate replacements contained known concentrations of ouabain for appraisal each against a tentatively selected concentration of the unknown substance. In this way the degree of restoration of the contractile response was obtained at multiple dose levels in staircase fashion. It was considered desirable to ignore any threshold effect, but rather to assess the potency of the drug on the basis of the whole dosage-response curve. Particular attention was paid to the middle limb of the log-dose-response curve which was approximately linear in its steepest part. After an adequate pilot experiment it was possible so closely to estimate the isodynamic equivalent of the unknown substance that a very small interval of log-dosage could be detected.

The methodology has been discussed in detail in the communication immediately preceding (1).

EXPERIMENTAL DATA. The crude experimental data obtained from nine muscles so studied are presented in table II of the communication immediately preceding this (1). These experiments give comparative responses for ouabain and digitoxin, respectively. In general, these responses (both for the standard

¹ For the pure substances used in this study the authors are indebted chiefly to Dr. K. K. Chen of the Eli Lilly Company. Additional material was contributed by John Wyeth Company.

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and the unknown) are recorded in pairs, each indicated at a stated titular dose. The titular dose represents the actual concentration of hydrated ouabain which was used. At each titular dose the true concentration of the unknown was the titular dose multiplied by E and the antilog of i . However, these hidden factors (which were established in planning the experiment) remained in abeyance until the data of each experiment had been recorded. In general, the photographic records were recorded at intervals over a period of some seven hours following the preliminary "fatiguing". Usually the photographic record represented a magnification of a hundred-fold, although at times a magnification of only 30-fold to 60-fold was adequate. Therefore, the actual magnitude of each record is unimportant, because the essential measurements for the unknown were the relative values compared with hydrated ouabain. These were carefully determined from the record with calipers and tabulated as discussed in a previous communication (4).

Before appraising each substance in terms of its companion responses to ouabain, the two regression coefficients were first tested for the significance of the slopes (5). In no instance was it clearly demonstrated that the slopes were different. Thereupon, the problem resolved itself into the assessment of the value for the intercept M between the two linear functions. The methods used were those of Bliss (6), Sherwood (7), and Snedecor (5). These authors have suggested several simplified methods which make such calculations very convenient provided that the same titular dose can be used for both standard and unknown at equal intervals of log-concentration. When the apparent potency was calculated in this fashion it was then necessary to correct this apparent potency for the hidden factor E which was introduced at the beginning of the experiment.

In the introductory or pilot experiments it sometimes turned out that the standard and unknown curves departed considerably from parallelism. This finding usually indicated that a poor estimate of the isodynamic equivalent had been made. As more familiarity was gained with each compound, curves so found tended to approximate each other closely and had similar slopes. In the ideal experiment the responses for the two substances, standard and unknown, turned out to be very nearly equivalent. The difference was calculated as a log-increment which could be translated back into a percentile change. From the percentile potency so determined the true equivalent concentration representing 1.0 milligram or unit of crystalline hydrated ouabain in the standard volume could be found. (See table I of the communication immediately preceding this (1).)

REPRODUCIBILITY OF DATA. In order to test the reliability of the procedure, five substances were studied by investigations a year apart. The comparative results, expressed simply as means, follow in table I. It will be observed that these independent studies yielded essentially the same results.

COMPARATIVE POTENCIES OF TWELVE GLYCOSIDES. Two types of comparisons have been made as follows.

(1) The relative potency obtained with the papillary muscle has been com-

pared with the mean lethal dose obtained by Chen (8) in intact cats. In three instances, also, Farah's results (9) are included.

(2) The relative potencies of the individual glycosides have been compared with one another.

TABLE I

Results of independent studies on relative equivalent dosage

GLYCOSIDE	STUDY 1946 (INVESTIGATORS W & B)		STUDY 1947 (INVESTIGATOR SCIARINI)	
	No. of expts.	Mean	No. of expts.	Mean
Convallotoxin.....	5	0.61	4	0.56
Digitoxin.....	5	2.2	4	2.6
Cerebroside.....	4	3.9	4	3.6
Thevetin.....	2	6.1	5	5.9
Uzarin	4	36.5	4	37.7

TABLE II

Ouabain-equivalence of digitoxin congeners

DRUG	RELATIVE VALUE OF MEAN LETHAL DOSE (CATS)*	EQUIVALENT DOSAGE FOR INOTROPIC RESPONSE, PAPILLARY MUSCLE (CATS)†	NUMBER OF ASSAYS
Convallotoxin.....	0.68	$0.60 \pm .04$	9
Cymarín.....	0.95	$0.90 \pm .06$	5
Calotropin.....	0.96	$0.91 \pm .11$	7
Ouabain, U.S.P. XII. ..	1.00 (arbitrary)	1.00 (arbitrary)	—
Scillaren A.....	1.26	$0.79 \pm .11$	5
Cerberin.....	1.27	$0.61 \pm .09$	4
Oleandrin.....	1.70	$1.29 \pm .14$	7
	1.3 (Farah)‡		
Digoxin... ..	1.99	$2.24 \pm .12$	9
	2.17 (Farah)‡		
Digitoxin... ..	2.80	$2.44 \pm .14$	9
	2.7 (Farah)‡		
Cerebroside. .	7.01	$3.74 \pm .34$	8
Thevetin...	7.67	$6.1 \pm .80$	7
Uzarin. . . .	39.5	37.1 ± 5.8	8

* Data of Chen (8), recalculated as per cent of ouabain \div 100.

† Reciprocals of potency, referred to ouabain as unity.

‡ Data of Maresh and Farah (9), recalculated.

As shown in table II, it seemed more convenient *not* to express potencies directly, but *rather* to calculate the relative dosage or concentration with hydrated ouabain (11%) arbitrarily set at unity. In re-calculating Chen's and Farah's results, therefore, each mean lethal dose was divided by the corresponding value for hydrated ouabain (i.e., 116 micrograms per kilogram). Similarly, in the case of the papillary muscle, the weight of any glycoside needed to reproduce the same response as *one* microgram of hydrated ouabain was calculated. In other

words, the reciprocals of relative potencies are shown. By this device direct comparisons are facilitated.

DISCUSSION. It will be observed from table II that the over-all picture of relative potency is surprisingly similar both for the lethal dose and the inotropic effect. The chief reversals in order are for Scillarin A and Cerberin. When one considers the many complicating factors in the intact animal,—e.g., distribution, destruction, excretion—it is perhaps striking that the general order of potency is preserved. Farah's method (9) has the advantage *for the present purpose* that the factor of time is minimized as it is in the technique for isolated myocardium. In short, both of these methods represent closer approaches to a steady or equilibrium state. Therefore, one would expect a closer correspondence with Farah's data than with those attained by the classical assay method.

The high relative potency of digitoxin disclosed by the inotropic response has been obtained also by Wedd and Blair (10) on strips of turtle ventricle.

It should be emphasized that the papillary muscle preparation is open to the same theoretical criticisms as all surviving tissues. In particular, the long period of 6 to 9 hours during which the myocardium is contracting in the usual experiment must be considered critically, to be sure that progressive deterioration of the tissue does not invalidate the results. These and similar considerations have been examined in the companion report which immediately precedes this (1).

SUMMARY

The inotropic effects of twelve pure "cardiac glycosides" upon isolated hypodynamic myocardium have been compared. The over-all comparison agrees closely with results reported for mean lethal dose in intact cats, although two compounds were displaced in order of sequence.

The authors wish to acknowledge helpful advice from Dr. McKeen Cattell.

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THE ACTION OF P-AMINOSALICYLIC ACID (PAS) IN EXPERIMENTAL TUBERCULOSIS

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The demonstration by Bernheim that sodium salicylate augmented the oxygen uptake of tubercle bacilli in a more or less specific manner (1, 2), thus suggesting the possibility that it or compounds of similar configuration may be important metabolites of the tubercle bacillus, prompted Lehman to study the antitubercular action of a large series of structurally related compounds. Using a bovine strain of tubercle bacilli he was able to show that of all the compounds studied p-amino salicylic acid (PAS) was the most active inhibitor *in vitro* (3). He further showed that the compound was not too toxic in experimental animals and its use in man was attended with apparent benefit (3, 4). Sievers (5) showed that the antibacterial action of PAS was specific for the tubercle bacillus since it inhibited the growth of the latter in the low concentration of 0.15 mg. per cent but it had no effect on growth of 19 species of non-acid-fast bacteria either pathogenic or non-pathogenic. Nor was it effective in protecting mice injected with pneumococci or with tetanus toxin.

Following these observations Youmans reported experiments indicating a tuberculostatic action of p-aminosalicylic acid in the order of 0.019–0.156 mg. per cent, that streptomycin resistant strains were equally sensitive to it, and that it had a suppressive effect on the disease in mice infected with tubercle bacilli when fed in a concentration of 1 per cent in the diet. At 2–4 per cent in the diet the compound was too toxic (6, 7).

The present work describes the toxicity of p-amino salicylic acid² in rats, guinea pigs and rabbits; its absorption, retention and excretion; and its therapeutic efficacy in experimental tuberculosis infections in guinea pigs and rabbits when used alone or in combination with streptomycin. The latter was attempted in view of the mutual potentiation of streptomycin and sulfone derivatives in guinea pig tuberculosis as previously reported (8).

EXPERIMENTAL. *Acute toxicity.* This is relatively low in all the animals studied. The intravenous injection of PAS as the sodium salt was tolerated in rats up to 2.0 gm. per kg. Higher doses were not attempted. The oral administration of PAS to guinea pigs in aqueous suspension with gum acacia and sufficient NaHCO_3 to neutralize the acid caused no mortality in doses of 2.0 gm./kg. and 30% mortality (3/10) when given in doses of 3.0 gm./kg. In rabbits 0.5 gm./kg. given intravenously as the sodium salt, or orally in aqueous suspension with gum acacia was well tolerated. Higher doses were not attempted.

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² We are indebted to Parke, Davis and Co. for generous supplies of this compound.

Chronic toxicity. Guinea pigs receiving the drug orally 0.5 gm./kg. daily, five days a week with a double dose on the fifth day until a total of 32 doses had been given had a mortality of 70% (7/10), thus suggesting cumulative action. Three of the 10 animals survived 36 doses. This may be seen in figure 1.

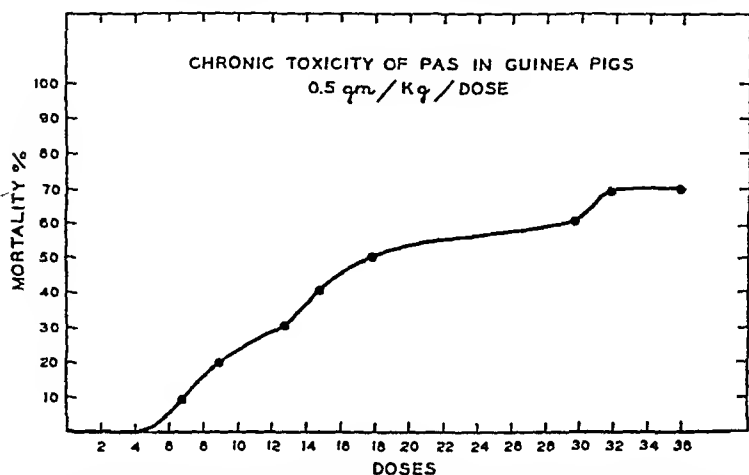


FIG. 1

Mortality rate of guinea pigs under continuous treatment with 0.5 gm./kg./day of PAS administered orally as a 10% aqueous suspension with gum acacia and sufficient NaHCO_3 to neutralize the acid.

TABLE I

Blood Levels Following Administration of PAS. (Average of 2-3 experiments)

ANIMAL	DOSE	ROUTE	BLOOD CONCENTRATION MG. PER CENT, HRS.						DIAZOTIZATION
			$\frac{1}{2}$	1	2	3	5	24	
Guinea pig	0.5 gm./kg.	oral	—	20	—	18	10	—	Direct
	1.0	oral	—	—	50	33	—	0	Direct
Rabbit	0.5	i.v.	16	18	12	—	0	0	Direct
			68	42	26	—	0	0	After hydrolysis in 0.2 N HCl at 100° C. one hour

The fate of the substance in the body is indicated from the data given in table I. The compound is well absorbed from the gastro intestinal tract, and is well retained for several hours. The concentration in the blood falls off more rapidly when given intravenously. Analysis of the urine of the rabbits referred to in table I showed that during the twenty four hours following the administration of the drug approximately 10 to 20% of the dose administered was excreted as the free compound and about 80% to 90% as the conjugated, probably acety-

lated compound³. On acidifying the urine with HCl, centrifugation and repeated washing of the centrifugate with water a crystalline material was obtained which on drying gave a sharp melting point of 225–226°C. The melting point of the PAS used was 150–151°C. This material gave no color on direct diazotization, but gave quantitative results after acid hydrolysis at 100°C. for one hour.

In like manner three guinea pigs receiving orally 156, 196 and 264 mg. PAS respectively (0.5 gm./kg.) excreted in the urine within 20 hours 124, 100 and 138 mg. respectively, or 51 to 79 per cent of the dose administered. Seventy to 87 per cent of that excreted in the urine was conjugated.

The *chemotherapeutic activity* of PAS in tuberculosis was studied in rabbits and guinea pigs, both alone and in combination with streptomycin.

Rabbit Experiments. Twenty four rabbits weighing 1.6 to 2.6 kg. were inoculated intravenously with 1 cc. of a suspension of a bovine strain (Ravenel) containing approximately 0.01 mg. moist weight of tubercle bacilli. The animals were divided into four equal groups. One group was kept as controls, the second group was treated with PAS, the third group was treated with streptomycin and the fourth group with PAS and streptomycin in the same doses as given in the second and third groups. The drug PAS was administered in doses of 0.2 gm./kg. intravenously daily for 7 days, then the dose was increased to 0.5 gm./kg. daily and administered orally to the termination of the experiment at 50 to 56 days after infection. The streptomycin was administered in doses of 20 mg./kg. twice daily, intravenously for the first 8 days and intramuscularly for the remainder of the experimental period. The animals were treated 5 days a week with a double dose on the fifth day. At the end of the experimental period the survivors were killed with chloroform, autopsied, and the extent of tuberculous involvement in the lungs, kidneys, liver, spleen and intestine noted and rated on the basis of 0 to 4. In doubtful cases histological sections were prepared and the lesions studied microscopically.⁴

The results of this experiment are summarized in table II. Of the untreated controls all the animals had slight to moderate or generalized tuberculosis of the lungs and kidneys with an average degree of tuberculosis for the group of 5.1. Of the animals treated with PAS one had no gross evidence of the disease. However, this animal died 31 days after infection, probably of drug toxicity. All the others in this group had sufficient involvement to give an average tuberculosis index of 3.0 for the group. Five of the animals treated with streptomycin had no lesions, and 1 had minimal lesions, while in the group treated with the combination all the animals were free from lesions. Some of the animals in the several groups had pitted livers, some cholangitis and one had ascites, all of which were probably due to coccidiosis.

³ Estimations were made by an adaptation of the Bratton and Marshall technique (9) using the Fisher electrophotometer with B 525 filter. The values given are only approximate. Diazotization and coupling of PAS may give erratic results. Freshly prepared aqueous solutions of PAS often fail to give any color, while solutions standing for several days at room temperature or solutions heated at 100° C. for 30–60 minutes yield quantitative linear curves when plotting absorption against concentration. In the case of urines or deproteinized blood filtrates preliminary beating may also be necessary if the results appear irregular.

⁴ For this we are indebted to Dr. W. T. S. Thorp of the Pathology Laboratory.

TABLE II

The Action of PAS Alone and in Combination with Streptomycin in Experimental Tuberculosis in Rabbits

Infecting dose .01 mg. tubercle bacilli, Ravenel bovine strain, intravenously

No.	WEIGHT		DAYS	TUBERCULOUS INVOLVEMENT						REMARKS
	Initial	Final		Lungs	Kidney	Liver	Spleen	In- testine	Total	
Controls										
1	2.0	2.4	50	3	2	0	0	0	5	Died
2	2.3	2.5	53	3	1	0	1	0	5	
3	1.7	2.0	53	2	1	0	0	0	3	
4	1.7	1.9	56	3	2	1	0	0	6	
5	1.7	1.9	36	3	2	0	1	0	6	
6	1.6	1.8	56	2	1	0	0	3	6	
PAS										
1	1.9	2.2	53	2	1	2	0	0	5	Pulmonary congestion Died. Liver necrosis Pitted liver
2	2.3	2.2	50	1	1	0	0	2	4	
3	2.4	2.5	53	2	1	0	0	0	3	
4	2.0	2.1	31	0	0	0	0	0	0	
5	2.4	2.8	56	1	1	0	1	0	3	
6	2.4	2.5	56	3	0	0	0	0	3	
Streptomycin										
1	1.6	2.0	53	0	0	0	0	0	0	Ascites, Pitted liver Pulmonary congestion Pulmonary congestion Traumatic paralysis, killed Cholangitis, pulmonary edema Pitted liver, pulmonary congestion
2	2.0	2.4	50	0	0	0	0	0	0	
3	1.8	2.2	53	0	0	0	1	0	1	
4	2.1	1.8	36	0	0	0	0	0	0	
5	1.9	2.0	56	0	0	0	0	0	0	
6	2.0	2.4	56	0	0	0	0	0	0	
Streptomycin + PAS										
1	2.3	2.3	53	0	0	0	0	0	0	Broncho-pneumonia Died. Pulmonary and hepatic congestion Cholangitis, pulmonary congestion
2	2.4	2.5	53	0	0	0	0	0	0	
3	2.1	2.3	50	0	0	0	0	0	0	
4	2.6	2.9	16	0	0	0	0	0	0	
5	2.1	2.4	56	0	0	0	0	0	0	
6	2.2	2.6	56	0	0	0	0	0	0	

These experiments demonstrate the therapeutic efficacy of streptomycin in experimental bovine infections and suggest a slight suppressive effect from PAS. No conclusions can be drawn regarding the efficacy of PAS as a supplementary

therapeutic agent to streptomycin since the dose of the antibiotic employed was large enough to afford nearly complete protection when used alone.

Experiments in guinea pigs. Forty male guinea pigs of about 300-350 gm. were inoculated intraperitoneally with 1 cc. of a bacillary suspension containing 0.5 mg. moist weight of the human strain tubercle bacilli A27. The series was divided into four equal groups, the first to serve as controls, the second for treatment with streptomycin, the third for treatment with PAS, and the fourth for treatment with both drugs at the same dose levels as in the preceding two groups. The streptomycin was administered in doses of 10 mg./kg. twice daily. The PAS was administered orally once daily, 0.5 gm./kg. the first 3 weeks, then on account of toxicity symptoms the dose was reduced to 0.3 gm./kg. for two weeks followed

TABLE III

The Action of PAS, Alone and in Combination with Streptomycin, in Experimental Guinea Pig Tuberculosis

Infecting dose 0.5 mg. human strain A27, i.p.

GUINEA PIG NO.	CONTROL		STREPTOMYCIN ¹		PAS		STREPTOMYCIN + PAS ¹	
	Survived days	T.B.	Survived days	T.B.	Survived days	T.B.	Survived days	T.B.
1	47	10	98	1	K ² 105	18	97	2
2	79	16	97	2	17	6	97	0
3	K 105	4	97	1	51	7	98	3
4	90	12	98	1	K 106	5	98	1
5	74	12	98	2	59	14	98	1
6	K 105	7	98	2	K 106	10	98	1
7	93	12	97	0	12	2	98	1
8	67	14	98	1	51	2	97	1
9	76	7	98	4	57	2	98	2
10	81	13	97	1	77	3	98	1
Number dead..	8		0		7		0	
Average T.B..		10.7		1.5		7.4 ³		1.3
C.E. ⁴			7.1		1.4		8.2	

¹ All killed, none died.

² K = killed.

³ Exclusive of No. 7.

⁴ Chemotherapeutic effectiveness (T.B. of controls/treated).

by 0.5 gm./kg. for the remainder of the experimental period. Treatment was begun the day after infection and continued for 11 weeks. Treatment was administered five days a week with a double dose on the fifth day. At 97-106 days after inoculation the survivors were killed with chloroform and autopsied. The degree and extent of tuberculous involvement in the omentum, spleen, liver, peritoneum and kidneys and lungs were rated on the basis of 0-4 with a possible maximum score of 20 for each animal. In doubtful cases histological sections were examined microscopically and spleen suspensions were inoculated aseptically into the groin of normal guinea pigs for PPD testing and autopsy 6 weeks later. Only the animals negative by all criteria were rated zero.

Table III shows the results of these experiments. The survival time and extent of tuberculous involvement (TB) are given for each animal of the four

groups. At the end of the experimental period eight of the controls were dead, seven in the PAS group were dead and none had died in the streptomycin or combined therapy groups. The average extent of tuberculous involvement in the control group was 10.7, the average for 9 in the PAS group was 7.4 (one animal died too early to have much tuberculosis), while those for the streptomycin and combined therapy groups were 1.5 and 1.3 respectively. The chemotherapeutic effectiveness (ratio of extent of tuberculous involvement in the controls to the treated groups) was 1.4 for PAS and 7.1 for streptomycin, showing the relatively low activity, if any, of PAS as compared with streptomycin. The chemotherapeutic effectiveness of combined therapy was 8.2, just a little more

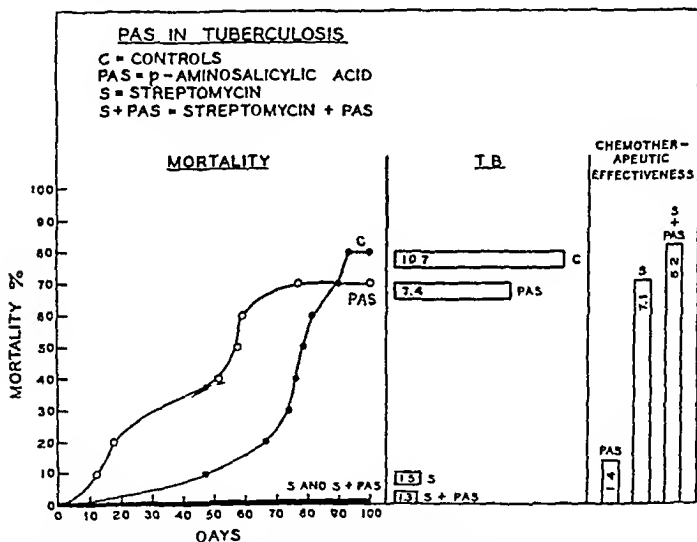


FIG. 2

Chemotherapeutic activity of PAS in experimental guinea pig tuberculosis, in comparison with that of streptomycin, and when used in combination with streptomycin.

than that of streptomycin alone and no greater than the sum of effects from the two drugs. (See also fig. 2.) Thus the chemotherapeutic efficacy of PAS in experimental guinea pig tuberculosis is slight or none compared with that of several sulfone derivatives previously reported, and unlike the sulfone derivatives PAS gives no indication of potentiation with streptomycin (8).

SUMMARY

p-Amino salicylic acid (PAS) was studied for its toxicity, fate in the body and chemotherapeutic efficacy in experimental tuberculosis, when used alone or in combination with streptomycin.

PAS has a relatively low acute toxicity, is well absorbed from the gastro-intestinal tract, is well retained in the body for 3-5 hours, is nearly completely

excreted in the urine within 24 hours, for the most part in the conjugated form, probably as the acetylated product. On continued administration there is evidence of cumulative action.

Pas has little therapeutic activity in rabbits infected with a bovine strain and in guinea pigs infected with a human strain of tubercle bacilli. Combined therapy with streptomycin showed no more than summation of effects, but gave no indication of potentiation, as was previously reported for sulfones and streptomycin (8).

Addendum. After completion of this work Feldman and associates (10) reported a favorable influence from PAS in tuberculous infections in guinea pigs. Their experimental approach was different and the results are not entirely comparable. They used a much smaller infecting dose, 1/500 of the dose we used, they used a different route of infection and they fed the drug in the diet so that the actual daily intake was not determined accurately but was estimated as 1.6 gm. per guinea pig per day. This would be approximately 3gm./kg. In the light of our work on the toxicity of PAS in guinea pigs this is far in excess of the tolerated dose. That their animals must have actually ingested much below their estimate receives strong support in their statement that "tests to determine the concentration of PAS in the blood of treated animals showed the drug to be present in a concentration of less than 0.5 mg. per 100 cc. of blood". Our work showed concentrations of 10-20 mg.% during a period of 5 hours following the oral administration of only 0.5 gm./kg. (see table I). Moreover, Feldman and associates did not use any standard of reference for the evaluation of the chemotherapeutic efficacy of PAS. We have evaluated its efficacy in comparison with streptomycin and on that basis this drug has little chemotherapeutic value when used alone and does not potentiate streptomycin when used with it.

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THE CURARIFORM ACTIVITY OF d-N-METHYL-ISOCHONDRODENDRINE AND d-O-METHYL-N-METHYL-ISOCHONDRODENDRINE

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Faltis (1) isolated d-isochondrodendrine² from "Pareira brava" and from crude commercial "bebeerine" and later (2) elucidated its structure. King (3) demonstrated that the dimethylether methiodide of this compound and that of neoprotocuridine, one of the active alkaloids in pot curare, are identical. Recently, Dutcher (4) found d-isochondrodendrine and d-O-methylisochondrodendrine in a curare prepared from Chondrodendron tomentosum, and indicated that the methiodides of both these compounds have some curariform activity. We obtained d-isochondrodendrine from "Pareira brava" by a modification of the method of King (5) and prepared the derivatives, d-N-methyl-isochondrodendrine and d-O-methyl-N-methyl-isochondrodendrine, as the iodide salts, by the method of Dutcher (4). These two compounds were compared with d-tubocurarine chloride pentahydrate and d-O-methyltubocurarine iodide trihydrate.

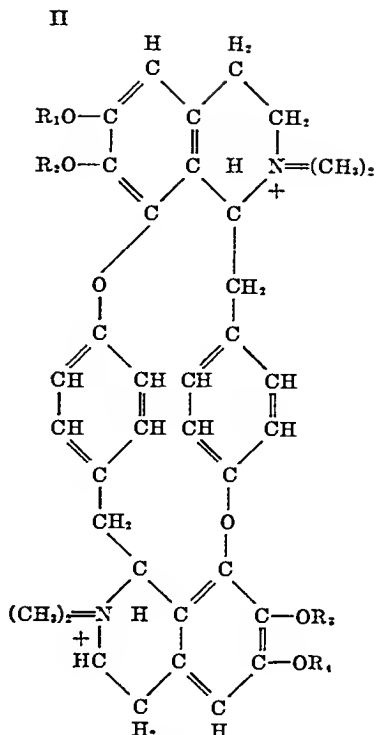
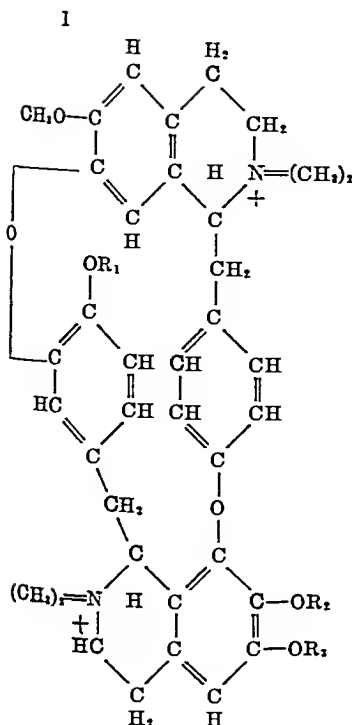
EXPERIMENTAL *Rats* The relative toxicity was first determined in 220 albino male rats (170-250 Grams). Solutions containing 0.25 mgm curariform ion per cc. were injected intraperitoneally and the lethal doses determined (see Table 1). Four to 10 minutes after receiving a lethal dose, the animals became limp and unable to walk, respiration stopped in an additional 3 to 6 minutes, and finally cardiac activity ceased. Unlike the quinine alkalioides (6), these compounds possess a very narrow margin between the dose producing a generalized lissive effect in rats and that producing respiratory failure, and we were unable to determine any so-called "paralytic dose" that would not also produce death in some animals.

Rabbits Solutions containing 0.25 mgm curariform ion per cc. were injected in fifteen seconds into the marginal ear vein of 40 single-sexed rabbits (1.7-3.0 kgm.) restrained in an enclosed box, and the dose producing head drop lasting a minimum of three minutes in half the animals determined (see Table 1). Later, the head drop cross-over assay of Holaday (7), as elaborated by Chase,

¹ Now at the University of Pennsylvania School of Medicine. Part of the material in this paper was presented before the American Society for Pharmacology and Experimental Therapeutics, Atlantic City, 1948. See *Federation Proceedings* 7: 248, 1948.

² Also known as d-isochondrodendrine and d-isodebeerine.

³ We are grateful to W. G. Bywater of S. B. Penick & Co., New York, for a large supply of milled "Pareira brava" prepared from the roots of four lots of Chondrodendron tomentosum.



Ia. d-Tubocurarine. $R_1 = H$, of R_2 and R_3 , one is H the other CH_3

Molecular weight 624.7. Used as the dihydrochloride-pentahydrate, Molecular weight 785.7, m. p. 268-9°C.

b. d-O-Methyltubocurarine. R_1 , R_2 , and R_3 are CH_3 . Molecular weight 652.8. Used as the dihydrochloride-trihydrate, Molecular weight 960.7, m. p. 257-8°C. (Anhydrous salt, m. p. 268-70°C)

IIa. d-N-Methyl-isochondrodendrine. R_1 or R_2 is CH_3 , the other H, R_3 or R_4 is H the other CH_3 . Molecular weight 624.7. Used as the dihydrochloride, Molecular weight 878.6, m. p. 280-1°C.

b. d-O-Methyl-N-methyl-isochondrodendrine. R_1 , R_2 , R_3 , and R_4 are CH_3 . Molecular weight 652.8. Used as the dihydrochloride, Molecular weight 906.6, m. p. 300-5°C.

Schmidt and Bhattacharya (8), in which increments of agent are added at fifteen second intervals until head drop occurs in all animals was utilized. Finally, the dose producing respiratory paralysis was approximated by this increment technique in one rabbit, this total dose injected in one minute or less in another two rabbits, and the dose decreased, if necessary, until only one of two rabbits died, and then the series expanded until the dose was found that killed five of ten rabbits

Cats Three hundred mgm of sodium barbital and 1 mgm. of atropine sulfate

per kgm. were administered intraperitoneally 60-90 minutes prior to operation in eight cats (1.9-3.3 kgm.). The femoral and sciatic nerves to one leg were cut and the peripheral end of the cut sciatic nerve stimulated with a motor driven interrupter for one tenth second with 6 volts 60 cycle half wave every ten seconds. The contractions of the gastrocnemius muscle were recorded on a soot kymograph with a weighted lever. Carotid blood pressure was recorded with the usual mercury manometer. The cats were mechanically oxygenated.

Fifty micrograms d-tubocurarine chloride pentahydrate per kgm. was given intravenously as a reference paralyzing dose. After the muscle returned to normal, various doses of the other agents were given until equivalent paralysis was produced (See Figure 1). The muscle could be partially paralyzed by this procedure for three to eight times before contractility no longer returned to normal after a given injection.

TABLE I

	d-TUBOCURARINE	d-O-METHYL-TUBOCURARINE	d-N-METHYL-ISOCHONDRODENDRINE	d-O-METHYL-N-METHYL-ISOCHONDRODENDRINE
Albino rats				
LD 50	0.22 (0.27)*	0.022 (0.032)	3.9 (5.5)	2.2 (3.0)
Rabbits				
Head Drop 50.....	0.10 (0.12)	0.011 (0.016)	1.8 (2.5)	0.4 (0.6)
Holaday Head Drop.	0.12 (0.15)	0.014 (0.020)	2.1 (2.9)	0.5 (0.7)
LD 50.. . . .	0.28 (0.35)	0.027 (0.040)	3.2 (4.5)	0.9 (1.3)
Cat gastrocnemius muscle				
Equivalent Paralysis....	0.04 (0.05)	0.005 (0.007)	0.7 (1.0)	0.14 (0.2)

* All doses given in mgm. of curariform ion per kgm. body weight. Dose of equivalent amount of salt used given in parenthesis. The standard errors of all the rat data lies within $\pm 4\%$ of the figures given, within $\pm 5\%$ for the rabbit data, $\pm 11\%$ for the cat data.

RESULTS. The d-N-methyl-isochondrodendrine ion is only about a twentieth, and the d-N-methyl-O-methyl-isochondrodendrine a fourth as paralyzant as d-tubocurarine, which in turn is only a tenth as active as the d-O-methyl-tubocurarine in the rabbit. With the cat preparation, the ratios are similar, but slightly smaller; i.e., the compounds do not differ so widely in activity. On the other hand, the ratios of the toxicities in the rat are larger, with the isochondrodendrine derivatives being relatively less toxic than the tubocurarine compounds.

The data for head drop in the rabbit by the Holaday technique is in good agreement with that quoted by Dutcher (4), although both our figures for d-tubocurarine are lower than those of Chase, Schmidt and Bhattacharya (8), which is apparently due to minor differences in the method of restraining the animals.

Like the tubocurarine compounds, these isochondrodendrine derivatives

have relatively little effect in intact animals other than lissive action on skeletal muscle. The rats did not show any signs that any of these agents had cholinergic activity (they did not sneeze or salivate or evidence chromodachyria or flush) as they do with some samples of crude curare. This was also true of the rabbits. Most of the cats had a transient fall in blood pressure following the administration of an effective dose of any of the agents, and this seemed to be related to the rapidity of injection and the concentration of the solution used. Since the blood pressure always returned to its normal level, these falls were not considered of great importance.

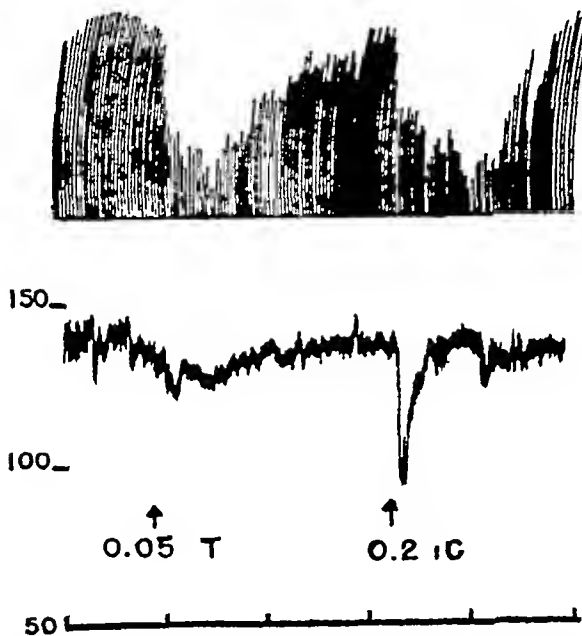


FIG. 1. 2.1 KG. MALE CAT

Gastrocnemius contractions, above, and blood pressure in mm Hg, below. 0.05 mgm. d-tubocurarine chloride pentahydrate per kgm. given at T and 0.2 mgm. d-O-methyl-N-methyl-isochondrodendrine iodide per kgm. given at IC. Time marks at 5 minute intervals.

DISCUSSION. d-Tubocurarine is a clinically necessary drug, but it is difficult to obtain and purify. Since isochondrodendrine is readily obtainable from the dried roots of many chondrodendron species (5) fairly widely distributed in nature and these easily prepared derivatives have the same desired curariform activity, it would seem reasonable to investigate these agents further with the object of their possible clinical introduction.

SUMMARY

d-N-Methyl-isochondrodendrine iodide and d-C-methyl-N-methyl-isochondrodendrine iodide were compared with d-tubocurarine chloride pentahydrate

and d-O-methyltubocurarine iodide trihydrate in rats, rabbits, and cats. d-N-Methyl-isochondrodendrine (as the ion) is about 1/20 and d-O-methyl-N-methyl-isochondrodendrine 1/4 as paralyzant as d-tubocurarine, which in turn is only 1/10 as active as d-O-methyltubocurarine. Like the tubocurarine compounds, these isochondrodendrine derivatives have relatively little effect in intact animals other than lissive action on skeletal muscles.

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